SHOUT
SHARE
IT

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory

FREDERICK B. BANG, Johns Hopkins University

EDWARD M. BERGER, Dartmouth College

STEPHEN C. BROWN, State University of New York at Albany

HARLYN O. HALVORSON, Brandeis University

J. B. JENNINGS, University of Leeds

FRANK J. LONGO, University of Iowa

GEORGE O. MACKIE, University of Victoria

JOEL L. ROSENBAUM, Yale University

HOWARD A. SCHNEIDERMAN, Monsanto Company

DOROTHY M. SKINNER, Oak Ridge National Laboratory

J. RICHARD WHITTAKER, Wistar Institute, Boston University Marine Program, and Marine Biological Laboratory

E. O. WILSON, Harvard University

GEORGE M. WOODWELL, Ecosystems Center, Marine Biological Laboratory

Editor: CHARLES B. METZ, University of Miami

VOLUME 160

FEBRUARY TO JUNE, 1981

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

The Biological Bulletin is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $10.00. Subscription per volume (three issues), $27.00 ($54.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and to Dr. Charles B. Metz, Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year.

The Biological Bulletin (ISSN 0006-3185)

Second-class-postage paid at Woods Hole, Mass., and additonal mailing offices.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1, February 1981

ALLEN DENNIS M., AND WENDY B. ALLEN
Seasonal dynamics of a leech-mysid shrimp interaction in a temperate salt marsh ... 1
BELLON-HUMBERT, CHANTAL, FRANÇOIS VAN HERP, GODFRIED E.C.M. STROLENBERG, AND J. MANUEL DENUCÉ
Histological and physiological aspects of the medulla externa X organ, a neurosecretory cell group in the eyestalk of *Palaemon serratus* Pennant (Crustacea, Decapoda, Natantia) ... 11
CIMBERG, ROBERT L.
Variability in brooding activity in the stalked barnacle *Pollicipes polymerus* ... 31
CLARK, KERRY B., KATHE R. JENSEN, HUGH M. STIRTS, AND CESAR FERMIN
Chloroplast symbiosis in a non-elysiid mollusc, *Costasiella lilianae* Marcus (Hermaeidae: Ascoglossa (=Sacoglossa): effects of temperature, light intensity, and starvation on carbon fixation rate ... 43
DURLIAT, MICHÈLE, AND ROGER VRANCKX
Action of various anticoagulants on hemolymphs of lobsters and spiny lobsters ... 55
GOVIND, C. K., T. W. BUDD, AND H. L. ATWOOD
Fiber composition and innervation patterns of the limb closer muscle in the lobster *Homarus americanus* ... 69
HAYES, PAUL F., AND R. WINSTON MENZEL
The reproductive cycle of early setting *Crassostrea virginica* (Gmelin) in the northern Gulf of Mexico, and its implications for population recruitment ... 80
HERMAN, WILLIAM S.
Studies on the adult reproductive diapause of the monarch butterfly, *Danaus plexippus* ... 89
JARAMILLO, EDUARDO, JORGE NAVARRO, AND JÜRGEN WINTER
The association between *Mytilus chilensis* Hupe (Bivalvia, Mytilidae) and *Edotea magellanica* Cunningham (Isopoda, Valvifera) in Southern Chile ... 107
KUMAR, T. PAVAN, R. RAMAMURTHI, AND K. SASIRA BABU
Circadian fluctuations in total protein and carbohydrate content in the slug *Laevicaulis alte* (Ferussac, 1821) ... 114
NICKLAS, NANCY L., AND RICHARD J. HOFFMANN
Apomictic parthenogenesis in a hermaphroditic terrestrial slug, *Deroceras laeve* (Müller) ... 123
STOECKER, DIANE, R. R. L. GUILLARD, AND RHONDA M. KAVEE
Selective predation by *Favella ehrenbergii* (Tintinnia) on and among dinoflagellates ... 136
STUNKARD, HORACE W.
The life history, developmental stages, and taxonomic relations of the digenetic trematode *Lasiotocus minutus* (Manter, 1931) Thomas, 1959 146
STUNKARD, HORACE W.
The morphology, life history, and systematic relations of *Lasiotocus elongatus* (Manter, 1931) Thomas, 1959 (Trematoda: Digenea) ... 155
SUBRAMONIAM, T.
Protandric hermaphroditism in a mole crab, *Emerita asiatica* (Decapoda:Anomura) ... 161

THORINGTON, GLYNE, AND LYNN MARGULIS
Hydra viridis: transfer of metabolites between *Hydra* and symbiotic algae 175
WOLCOTT, THOMAS G.
Inhaling without ribs: The problem of suction in soft-bodied invertebrates 189

NO. 2, APRIL 1981

Invited paper

JACKLET, JON W.
Circadian timing by endogenous oscillators in the nervous system: toward cellular mechanisms 199
ECKBERG, WILLIAM R.
An ultrastructural analysis of cytoplasmic localization in *Chaetopterus pergamentaceus* 228
FÄNGE, RAGNAR, AND ARTUR MATTISSON
The lymphomyeloid (hemopoietic) system of the Atlantic nurse shark, *Ginglymostoma cirratum* 240
GRIFFITH, ROBERT W.
Composition of the blood serum of deep-sea fishes 250
HERNANDORENA, A.
Metabolic significance of the dietary CMP requirement in relation to *Artemia* morphogenesis 265
HILL, BURKE
Respiratory adaptations of three species of *Upogebia* (Thalassinidea, Crustacea) with special reference to low tide periods 272
JOHNSTON, I. S., P. L. JOKIEL, C. H. BIGGER, AND W. H. HILDEMANN
The influence of temperature on the kinetics of allograft reactions in a tropical sponge and a reef coral 280
LASKER, HOWARD R.
Phenotypic variation in the coral *Montastrea cavernosa* and its effects on colony energetics 292
MARTIN, VICKI JOAN, AND MARY BETH THOMAS
The origin of the nervous system in *Pennaria tiarella*, as revealed by treatment with colchicine 303
SAIGUSA, MASAYUKI
Adaptive significance of a semilunar rhythm in the terrestrial crab *Sesarma* 311
SAWYER, ROY T., FRANCOIS LEPONT, DUNCAN K. STUART, AND ANDREW P. KRAMER
Growth and reproduction of the giant glossiphoniid leech *Haementeria ghilianii* 322
SHUMWAY, SANDRA E.
Factors affecting oxygen consumption in the marine pulmonate *Amphibola crenata* (Gmelin, 1791) 332

NO. 3, JUNE 1981

ARMSTRONG, D. A., K. STRANGE, J. CROWE, A. KNIGHT, AND M. SIMMONS
High salinity acclimation by the prawn *Macrobrachium rosenbergii*: uptake of exogenous ammonia and changes in endogenous nitrogen compounds 349
COLLIER, J. R.
Protein synthesis in the polar lobe and lobeless egg of *Ilyanassa obsoleta* 366
DANIEL, THOMAS L.
Fish mucus: *In situ* measurements of polymer drag reduction 376

CONTENTS

DeRosa, Y. Susan, and W. Otto Friesen
Morphology of leech sensilla: observations with the scanning electron microscope 383
Franz, D. R., E. K. Worley, and A. S. Merrill
Distribution patterns of common seastars of the Middle Atlantic continental shelf of the Northwest Atlantic (Gulf of Maine to Cape Hatteras) 394
Goto, Taichiro, and M. Yoshida
Oriented light reactions of the arrow worm *Sagitta crassa* Tokioka 419
Sarsig, Beth L., and Georgia E. Lesh-Laurie
Bud separation in *Hydra oligactis* 431
Index to Volume 160 438

Volume 160 Number 1

THE
BIOLOGICAL BULLETIN

Marine Biological Laboratory
LIBRARY
APR 3 1981
Woods Hole, Mass.

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory
FREDERICK B. BANG, Johns Hopkins University
EDWARD M. BERGER, Dartmouth College
STEPHEN C. BROWN, State University of New York at Albany
HARLYN O. HALVORSON, Brandeis University
J. B. JENNINGS, University of Leeds
FRANK J. LONGO, University of Iowa
GEORGE O. MACKIE, University of Victoria
JOEL L. ROSENBAUM, Yale University
HOWARD A. SCHNEIDERMAN, Monsanto Company
DOROTHY M. SKINNER, Oak Ridge National Laboratory
J. RICHARD WHITTAKER, Wistar Institute of Anatomy and Biology
E. O. WILSON, Harvard University
GEORGE M. WOODWELL, Ecosystems Center, Marine Biological Laboratory

Managing Editor: CHARLES B. METZ, University of Miami

FEBRUARY, 1981

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $10.00. Subscription per volume (three issues), $27.00 (this is $54.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor; or Susan Schwartz, assistant editor, at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and at the Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year.

Copyright © 1981, by the Marine Biological Laboratory

Second-class postage paid at Woods Hole, Mass., and additional mailing offices.
ISSN 0006-3185

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978). Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the

Continued on Cover Three

Reference: *Biol. Bull.*, **160:** 1–10. (February, 1981)

SEASONAL DYNAMICS OF A LEECH-MYSID SHRIMP INTERACTION IN A TEMPERATE SALT MARSH

DENNIS M. ALLEN[1] AND WENDY B. ALLEN[1]

Dept. of Biology and The Wetlands Institute, Lehigh University, Bethlehem, PA 18015

ABSTRACT

The spatial and temporal distributions and aspects of the life history of the marine leech, *Mysidobdella borealis*, were studied in a salt marsh embayment and adjacent ocean area in southern New Jersey, U. S. A. Mysid hosts, *Neomysis americana*, occurred in all epibenthic sled collections, but leeches were only collected in the embayment in winter and spring. Leeches were in the cooler ocean areas during the summer. The recurrence of *M. borealis* in the embayment in November or December coincided with the migration of large mysids from the ocean. Leeches reproduced in the marsh each spring, but no life stages were collected after embayment temperatures reached 20°C. Laboratory experiments demonstrated that adult and newly hatched *M. borealis* were killed by long exposures to temperatures greater than 20°C. The recruitment and survival of the boreal-arctic leech in estuaries at the southern extent of its geographical range is determined by host migration and water temperature.

INTRODUCTION

Marine leeches have been reported from a variety of vertebrate and invertebrate hosts, but relationships between the spatial and temporal distributions of leech and host populations are not well known. Leeches of the family Piscicolidae are most often associated with fishes, but invertebrates, especially crustaceans, also serve as at least temporary hosts (Meyer and Barden, 1955). Decapod crabs often are the substrates on which leeches deposit cocoons after leaving their primary fish hosts (Moore and Meyer, 1951; Daniels and Sawyer, 1975). Other crustacean hosts include decapod shrimps (Selensky, 1923), amphipods (Epshtein, 1959), isopods (Sawyer and White, 1969), and mysids (Selensky, 1927; Dukina *et al.*, 1974).

Burreson and Allen (1978) described the anatomy, geographical range, and some aspects of the biology of the marine leech *Mysidobdella borealis* (Johansson) in the western North Atlantic. This leech has been reported from three mysids, *Mysis oculata* (Fabricius) in arctic seas (Selensky, 1927; Vasileyev, 1939; Epshtein, 1961), and *Mysis stenolepis* Smith and *Neomysis americana* Smith on the east coast of North America (Burreson and Allen, 1978).

The only known host of *M. borealis* on the coast of southern New Jersey is *N. americana*. Extensive laboratory tests failed to identify alternative hosts and various observations indicated that the leech was an obligate parasite which fed on the mysid (Burreson and Allen, 1978). Leeches introduced into aquaria containing *N. americana* swam until they made contact with a mysid and then remained attached. Adult leeches only left their hosts to deposit cocoons on hard substrates, and juvenile

Received March 23, 1980; Accepted October 17, 1980.

Abbreviations: UB, upper embayment; LB, lower embayment.

[1] Present Address: Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina, Georgetown, SC 29440.

M. borealis attached to *N. americana* within hours of hatching (Burreson and Allen, 1978).

The intent of the present study was to determine the seasonal distribution, life history, and physiological tolerances of *M. borealis* near the southern extent of its range and to examine the relationship between the population dynamics of the leech and the population dynamics and distribution of the mysid host, *N. americana*.

MATERIALS AND METHODS

Hereford Inlet (38°58′–39°06′ N, 74°44′–74°48′ W) includes a tidal embayment flanked on the east by a barrier island and on the west by extensive *Spartina alterniflora* Loisel marshes and shallow lagoons. Five sampling sites were along the major axis of tidal flow within the embayment and two were in the ocean (Fig. 1). Stations 1 and 2 had irregular mud bottoms (6–8 m) with accumulations of marsh detritus and macroalgae characteristic of the upper embayment (UB). Station 3 traversed a sandy basin (7–10 m) at the confluence of two major waterways. Stations 4 and 5 had rippled sand bottoms (5–8 m) typical of the current-scoured areas of the lower embayment (LB). Stations 6 and 7 were outside of the sand shoals at the mouth of Hereford Inlet and had flat sand bottoms (10–14 m) representative of the shallow ocean area.

Semidiurnal tides extend to the center of Great Sound (Fig. 1) and prevent vertical stratification of temperature, salinity, or dissolved oxygen in the embayment. Temperature gradients are established along the study transect during the spring and fall as a result of differential rates of change for the shallow sounds and coastal waters. Figure 2 shows the bottom water temperature curves for station 1 and 7 and demonstrates the lag in the warming of ocean waters during early summer. Little fresh water flows into Hereford Inlet and salinities remained 28–32‰ in the major waterways.

Leeches attached to their hosts, specimens of *N. americana*, were collected with an epibenthic sled designed to capture small organisms within 30 cm of the bottom. A rectangular steel frame (51 × 30 cm), which oriented the mouth of a #2 mesh (365 μ) Nytex net perpendicular to the bottom, was mounted on a horizontal frame which slid over the bottom on three skis. Preliminary studies demonstrated that towing the sled in the direction of the tidal current was the most effective way to collect mysids, which orient and swim into tidal currents near the bottom. Five-minute tows from a boat just making headway with the tidal current traversed approximately 100 m of bottom. Surface and bottom water temperatures and salinities were measured with an induction salinometer immediately after each tow.

The seasonal occurrence of *M. borealis* was determined from a series of 80 cruises between June 1975 and June 1977, in which at least stations 1–5 (Fig. 1) were sampled sequentially beginning at station 1 approximately 1.5 h after slack tide. Cruises were made at intervals of 7–10 days and a total of 424 collections taken. Each sample was preserved with buffered formalin immediately after collection. In the laboratory, the entire sample was usually enumerated, but collections which contained large volumes of detritus or organisms were split with a cylindrical plexiglass plankton splitter until a workable portion was obtained. All sorting and counting was done at 10.5× with a binocular zoom microscope.

Total numbers of *M. borealis* were recorded for each collection. Since preservative was added to the live samples in this series, the leeches were usually fixed in a twisted configuration, and total lengths could not be measured accurately. Leeches which were less than 3 mm were considered juveniles, those 3–5 mm were

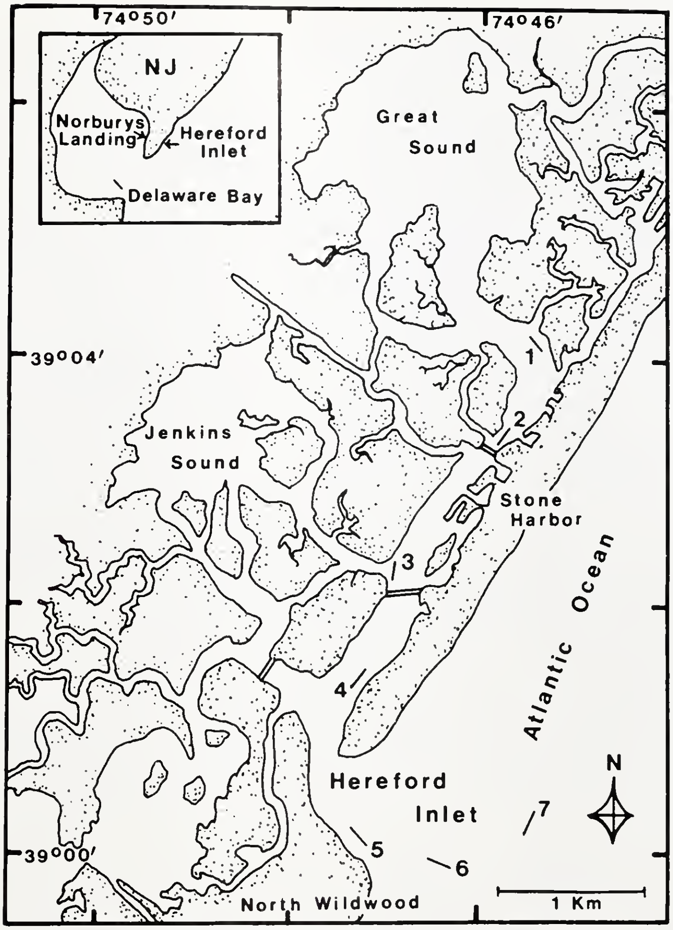

74°50′
74°46′
NJ
Norburys Landing
Hereford Inlet
Delaware Bay
Great Sound
39°04′
1
2
Jenkins Sound
Stone Harbor
3
Atlantic Ocean
4
N
Hereford Inlet
7
5
6
39°00′
North Wildwood
1 Km

FIGURE 1. Location of sampling stations 1–7 at Hereford Inlet and Norburys Landing, New Jersey. Lines at the stations represent the orientations and approximate distances of sled tows.

small adults, and those larger than 5 mm were adults. Leeches collected in New Jersey waters were protandric and never exceeded 12 mm. Testisacs were partially atrophied in sectioned leeches over 7 mm in length, and the largest individuals possessed vestigal testisacs and mature ovisacs (Burreson and Allen, 1978).

The length frequency distribution of the leech population and the variability in the ratio of total numbers of leeches to total numbers of mysid hosts was determined in a series of consecutive tows at station 2 on four cruises. The collections were returned to the laboratory within an hour and most leeches remained swimming near the surface of the sample jar. All *M. borealis* were removed, then relaxed and straightened in dilute ethanol before being preserved in formalin. Leeches were measured to the nearest 0.1 mm with an ocular micrometer.

About 300 supplementary collections made with epibenthic sleds and plankton nets between March 1974 and June 1978 were also examined for *M. borealis*. These samples were usually taken in areas different from those in the regular sampling program in Hereford Inlet and included many night collections.

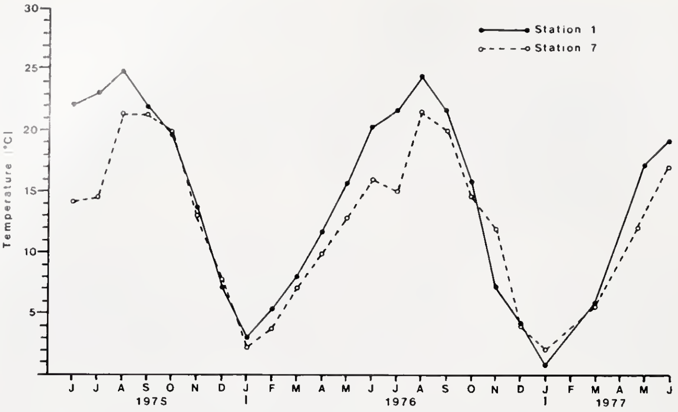


FIGURE 2. Mean monthly bottom water temperatures at UB station 1 and ocean station 7 from June 1975 to June 1977.

Collections were also made on the intertidal flats of Delaware Bay at Norburys Landing, New Jersey (Fig. 1). Samples were taken with hand-held sweep nets in shallow water every 1–3 days from late March to early June 1978. Salinities varied between 18 and 27‰ and temperatures from 4 to 22°C.

M. borealis specimens were maintained in a running sea water system from December through May each year. The swimming and attachment behavior of the leeches were observed. Small groups of leeches were isolated with mysid hosts in static aquaria at different temperatures for observations on reproduction and longevity.

Experiments were conducted to determine the temperature and salinity tolerances of *M. borealis.* Plastic containers, each with 350 ml of water at one of five salinities (0, 10, 15, 20, and 30‰) were placed into constant temperature baths and allowed to equilibrate for at least 12 h. Tests were run at six temperatures (0, 10, 15, 20, 22, and 25°C) and all temperature-salinity combinations were tested two or three times. Ten adult leeches and twelve mature *N. americana* were placed in each container without prior acclimation, and percentage survival was recorded each day for 10 days.

RESULTS

Mysidobdella borealis occurred in 120 of the 424 epibenthic sled collections made in the regular sampling program. The mysid host, *Neomysis americana*, was in all samples. Ratios of leeches to mysids greater than 1:100 were found in 20 of the 120 collections in which leeches occurred. The highest incidence was 9.7:100 on 21 March 1977, at station 1. The greatest number of leeches taken in a 5-min tow was 744 (with 16,648 mysids) on 26 December 1975.

The mean monthly values for numbers of *M. borealis* per 1000 *N. americana* (Table I) indicate that leeches were most abundant from December to March or April. Leeches were seldom collected within the embayment during the summer or fall, even though *N. americana* was most abundant in the study area from June to October (Allen, 1978). Juvenile *M. borealis* were occasionally collected at the LB stations in July and August 1975; however, small leeches (3–5 mm) were consistently collected during this period at the ocean stations where water temperatures remained near 15°C. No leeches were collected at any station from mid-

August to mid-December 1975, and only a few individuals occurred at the ocean stations from early August to mid-November 1976 (Table I).

The recurrence of leeches in the study area each winter was first detected at the ocean stations. During the following weeks abrupt increases were observed in the embayment. Table II shows the results of cruises made before and after the first occurrence of leeches in the UB. Significant increases in total numbers, mean length, and length of the largest mature *N. americana* at UB stations 1 and 2 on 17 December 1975, coincided with the first occurrence of leeches in the UB since the previous spring. A similar but less distinct pattern of recurrence of large mysids and leeches was observed on 19 November 1976 (Table II).

A comparison of the abundance and size distribution of *M. borealis* at UB station 2 on four sampling dates during the 1977–78 infestation is shown in Table III. Leeches did not occur in any of the six replicate samplings on 12 October and supplementary sampling did not reveal leeches in the embayment until 21 December. At this time, leeches were associated with large overwintering mysids not found at any embayment stations the previous week. Collections on 23 December contained both juvenile and sexually mature leeches, but small adults dominated. Mean length and the length of the largest leeches had increased on 21 February and again on 24 April.

The seasonal pattern of occurrence of *M. borealis* on the extensive flats of Delaware Bay at Norburys Landing was similar to that in Hereford Inlet. Leeches

TABLE I

Mean monthly numbers of M. borealis *per 1000* N. americana *in epibenthic-sled collections made at stations 1–7 in Hereford Inlet from June 1975 to June 1977. Each value is based on 2–5 collections for which a ratio of total leeches to total mysids was determined. Dashes indicate that no samples were taken.*

Station	1	2	3	4	5	6	7
Jun 1975	0	0	0	0	0	—	—
Jul	0	0	<1	0	<1	0	<1
Aug	0	0	0	0	0	0	<1
Sep	0	0	0	0	0	0	0
Oct	0	0	0	0	0	0	0
Nov	0	0	0	0	0	0	0
Dec	16	7	3	4	14	2	<1
Jan 1976	2	6	2	9	6	1	3
Feb	1	<1	4	1	1	<1	1
Mar	1	3	3	16	8	4	1
Apr	1	<1	7	3	7	4	5
May	4	<1	0	0	0	<1	<1
Jun	0	<1	0	0	<1	<1	4
Jul	0	0	<1	0	<1	<1	2
Aug	0	0	0	0	0	<1	0
Sep	0	0	0	0	0	0	<1
Oct	0	0	0	0	0	0	0
Nov	0	<1	<1	<1	<1	<1	<1
Dec	3	5	<1	1	6	—	—
Jan 1977	1	3	6	5	2	—	—
Feb	—	—	—	—	—	—	—
Mar	36	11	3	11	6	10	<1
Apr	—	—	—	—	—	—	—
May	1	0	0	4	4	1	1
Jun	<1	<1	1	1	0	0	1

TABLE II

Results of collections made at UB stations 1 and 2 during the period of leech recurrence in 1975 and 1976. L/M is the number of M. borealis *per 1000* N. americana *based on the ratio of total leeches per total mysids for each collection. nM is the total number of* N. americana. $\bar{X}$ *is the mean total length (mm) of mature* N. americana ± *standard deviation.* ^{X}*min*–^{X}*max is the range in total length (mm) of* N. americana. *Temperature is the same for both stations.*

		Station 1				Station 2			
Date	Temp.	L/M	nM	$\bar{X}$ ± SD	^{X}min–^{X}max	L/M	nM	$\bar{X}$ ± SD	^{X}min–^{X}max
3 Nov 1975	16°C	0	7080	9.6 ± 0.6	8.8–10.5	0	4169	9.9 ± 0.6	8.8–11.1
17 Nov 1975	12°	0	64	—	—	0	69	11.8 ± 0.8	10.9–13.0
2 Dec 1975	10°	0	105	10.9 ± 1.3	10.3–14.3	0	47	—	—
17 Dec 1975	8°	4	4885	14.5 ± 1.7	12.6–17.4	1	4200	15.3 ± 1.7	12.8–17.6
16 Jan 1976	3°	36	2082	14.7 ± 1.7	12.8–17.8	9	956	15.8 ± 1.9	13.2–17.9
25 Oct 1976	14°C	0	15325	10.5 ± 0.6	9.2–11.1	0	20832	10.5 ± 0.9	9.5–11.8
3 Nov 1976	9°	0	2936	10.3 ± 0.4	9.9–10.9	0	3198	10.5 ± 0.6	9.0–11.6
19 Nov 1976	7°	0	351	12.0 ± 1.0	10.7–13.4	1	6364	11.3 ± 1.3	9.7–14.1
6 Dec 1976	4°	3	10220	13.0 ± 0.6	11.3–14.1	5	15480	13.2 ± 0.6	12.4–14.1
6 Jan 1977	1°	1	8424	13.0 ± 0.9	10.1–16.4	3	12416	12.4 ± 1.3	10.3–14.1

were associated with *N. americana* during the coldest months and disappeared by early summer. In March, large leeches were attached to large mysids and few juveniles of either species were collected. After the peak brood release and die-off of large overwintering mysids in mid-April, few large leeches were collected. By early May, some large leeches and mysids remained, but both populations were dominated by very small individuals. Samples taken in mid-May yielded thousands of juvenile mysids (<5 mm) heavily infested with very small, apparently newly hatched leeches (<3 mm). Early June collections showed young mysids still abundant, but leeches virtually absent. No leeches were collected at temperatures higher than 21°C.

In the laboratory, *M. borealis* copulated and deposited cocoons from January to May (5–21°C), but the most intense activity was in March and April at 15°C. Leeches copulated while attached to mysid hosts. Adult leeches left their hosts to deposit their 0.5 mm cocoons on the seams of container walls and, in some cases, in depressions on the surfaces of sand grains. All leeches died within a few days after depositing cocoons. Increasing water temperature induced groups of cold-acclimated leeches to deposit cocoons within 10 days. Cocoons hatched in about

TABLE III

Results of four series of replicate collections at UB station 2 during the 1977–78 infestation of M. borealis. *N is the number of replicate tows. L/M is mean number of* M. borealis *per 1000* N. americana ± *standard deviation (coefficient of variation). The ratio is based on total leeches per total mysids determined for each replicate.* $\bar{Y}$ *is the mean length of* M. borealis ± *standard deviation.* ^{Y}*min*–^{Y}*max is the range in total length of* M. borealis. *% JUV is the percentage of the sample population that was less than 3 mm in length.*

	T°C	S (‰)	N	L/M ± SD (CV)	$\bar{Y}$ ± SD	^{Y}min–^{Y}max	% JUV
12 Oct 1977	16.1	30.1	6	0	—	—	—
23 Dec 1977	5.0	29.8	7	11 ± 2 (18%)	4.6 ± 0.8	2.0–6.2	1
21 Feb 1978	3.1	30.6	6	5 ± 1 (20%)	6.1 ± 1.0	2.3–8.4	6
24 Apr 1978	10.4	30.1	6	23 ± 4 (17%)	8.3 ± 1.1	2.3–10.1	14

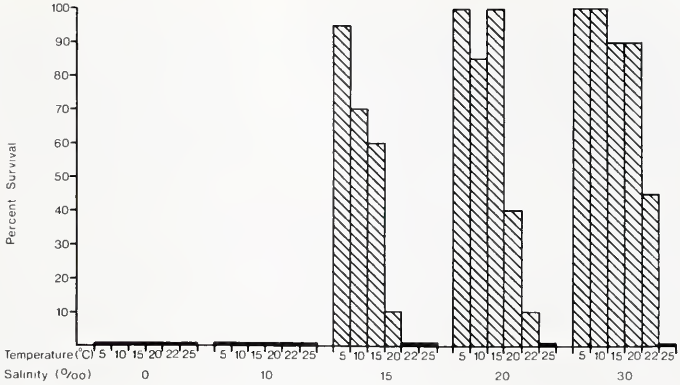


FIGURE 3. Mean values for the survival of adult *Mysidobdella borealis* in 30 combinations of temperature and salinity after 10 days exposure.

4 weeks at 20°C and in about 14 weeks at 7°C. We did not collect cocoons in the wild. Juvenile leeches (1.8–2.2 mm) usually attached themselves to *N. americana* within hours of emerging from cocoons. No young attached leeches survived more than 8 days at 20°C, but some juveniles remained attached to refrigerated mysids (7°C) for almost 6 months. These leeches were 2.7–4.4 mm in length when the experiment ended in late November.

Laboratory experiments indicated that *M. borealis* was intolerant of low salinities and high temperatures (Fig. 3). All leeches died in test salinities of 0 and 10‰ at all temperatures, and at 25°C at any salinity. Leeches survived best in combinations of high salinity and low temperature; *M. borealis* maintained below 10°C and at 30‰ in the laboratory usually survived for months.

DISCUSSION

The marine leech, *M. borealis*, suddenly occurred in sled collections in the salt marsh embayment at Hereford Inlet, New Jersey, each winter and persisted until early summer. The mysid host, *N. americana*, was ubiquitous in the major waterways throughout the year, but leeches were not associated with warm-water mysid populations. In New Hampshire, *M. borealis* was found only from December through March even though mysids were present all year (Burreson and Allen, 1978). Similar distributions of the leech and mysid were found in Barnegat Bay, New Jersey, where *M. borealis* occurred in 65 out of 307 samples from November 1975 to June 1976, but did not occur in any of the 224 collections taken from July through October (Sandine *et al.*, 1977).

The occurrence of leeches from late fall to early summer and their virtual absence during the warmest months has been reported for many temperate species (Thompson, 1927; Hoffman, 1955; Becker and Katz, 1965; Gibson and Tong, 1969; Halvorsen, 1971 and 1972; Sawyer and Hammond, 1973). Two alternative explanations for the warm-season absence of the leeches can be considered: (1) Life history patterns and behavior may enable summer populations to remain inconspicuous until the fall or (2) local populations die or migrate from the study area, then reappear in the fall. Sawyer and Hammond (1973) provide an example of the first alternative in their suggestion that *Calliobdella carolinensis* Sawyer and

Chamberlain embryos and juveniles escape detection during their slow summer development. Gibson and Tong (1969) reported that cocoons of *Oceanobdella blennii* (Knight-Jones) were capable of oversummering, so that reproductive structures deposited in the spring hatched 7 or 8 months later. In the case of *M. borealis* in Hereford Inlet, however, the second alternative must be considered.

When *M. borealis* was first collected in the embayment each winter, both juveniles and sexually mature individuals were present. Young leeches emerged from cocoons at about 2 mm and, if the small leeches in these collections originated from an oversummering population in the embayment, either the cocoons took a minimum of 6 months to hatch, or young hatched in the summer did not grow at all. Cocoons deposited in the laboratory hatched in less than 1 month at 20°C, and the juvenile leeches which emerged died within 8 days at this temperature. Laboratory experiments indicated that both juvenile and adult leeches were intolerant of warm temperatures and could not survive in the embayment at temperatures characteristic of the area from May to November.

Populations of *M. borealis* appeared to be introduced to the embayment each winter with an inshore migration of mysid hosts. As water temperatures decreased to about 10°C each fall, leeches and large mysids appeared in collections outside of Hereford Inlet. During the next 1–2 weeks, leeches appeared in the UB. Their recurrence coincided with major changes in the abundance and length frequency structure of the resident mysid population. Changes in the population structure of the mysids could not be explained by the growth of mysids already present in the UB, because the growth rate of *N. americana* at 10°C is about 1.0 mm/month (Pezzack and Corey, 1979) and the mean length of mysids collected in the UB increased by 3.6 mm between 2 and 17 December 1975. Increases in abundance and other evidence suggests a migration of large mysids from outside of the embayment (Allen, 1978).

Seasonal migrations of shallow water mysids have been reported from Europe (Tattersall, 1938; Vorstman, 1951; Holthuis, 1954; Kinne, 1955; Mauchline, 1967; and Hesthagen, 1973), South America (Almeida Prado, 1973), Australia (Hodge, 1963), and Russia (Zmudzinski, 1967). Migrations of *N. americana* have not been reported, but seasonal changes in distribution have been suggested by Black (1956), Hulburt (1957), and Herman (1963). Amaratunga and Corey (1975) described seasonal migrations of *Mysis stenolepis* in Passamaquoddy Bay, Canada, and the presence of *M. borealis* with this mysid host in shallow water during the summer may be related to the migration of the mysid from deep overwintering areas (Burreson and Allen, 1978).

M. borealis has been reported from the mysid, *Mysis oculata*, in the White Sea near Russia (Selensky, 1927), the Kara Sea and Aleutian Islands (Vasileyev, 1939), and Greenland (Epshtein, 1961) where cold, high salinity waters prevail. The seasonal occurrence of *M. borealis* as far south in the western North Atlantic as the shallow coastal areas of New Jersey and Delaware Bay is probably regulated by water temperature. Leeches which suddenly appear in the embayment each winter probably originate from a population associated with *N. americana* in deeper ocean areas. *N. americana* has been collected near the edge of the continental shelf at 232 m (Wigley and Burns, 1971) and is abundant in the ocean off New Jersey where water temperatures are always less than 20°C. Thus, migrating mysids function as dispersal agents for *M. borealis* by extending its distribution to coastal embayments and estuaries, but the inability of this arctic species to survive summer conditions prevents *M. borealis* from becoming a permanent member of the temperate salt marsh fauna.

ACKNOWLEDGMENTS

We thank Dr. Eugene M. Burreson for valuable discussion about the interaction and Dr. Sidney S. Herman for support in the field and laboratory studies at The Wetlands Institute. The financial support of the J. S. Noyes Foundation and the Huber Foundation is gratefully acknowledged.

LITERATURE CITED

ALLEN, D. M. 1978. *Population dynamics, spatial and temporal distributions of mysid crustaceans in a temperate marsh estuary.* Ph.D. Dissertation, Lehigh University, Bethlehem, Pennsylvania. 157 pp.

ALMEIDA PRADO, M. S. DE. 1973. Distribution of Mysidacea (Crustacea) in the Cananeia Region. *Biol. Zool. Biol. Mar., N. S.* **30:** 395–417.

AMARATUNGA, T., AND S. S. COREY. 1975. Life history of *Mysis stenolepis* Smith (Crustacea, Mysidacea). *Can. J. Zool.* **53:** 942–952.

BECKER, C. D., AND M. KATZ. 1965. Distribution, ecology, and biology of the salmonid leech *Piscicola salmositica* (Rhyncobdellidae: Piscicolidae). *J. Fish. Res. Bd. Can.* **22:** 1175–1195.

BLACK, W. F. 1956. *The Mysidacea of the Bras d'Or Lakes.* Ph.D. Dissertation. McGill University, Montreal, Canada. 210 pp.

BURRESON, E. M., AND D. M. ALLEN. 1978. Morphology and biology of *Mysidobdella borealis* (Johansson) comb. n. (Hirudinea: Piscicolidae) from mysids in the western North Atlantic. *J. Parisitol.* **64:** 1082–1091.

DANIELS, B. A., AND R. T. SAWYER. 1975. The biology of the leech *Myzobdella lugubris* infesting blue crabs and catfish. *Biol. Bull.*, **148:** 193–198.

DUKINA, V. V., V. S. TVOROVSKII, AND V. M. EPSHTEIN. 1974. The finding of the fish leech *Caspiobdella fadejevi* on shrimps (mysids) in the Pechenezh Reservoir. *Zool. Zh.*, **53(9):** 1413–1414.

EPSHTEIN, V. M. 1959. On the taxonomy, mode of life and origin of the endemic Baikal leech *Trachelobdella torquata* (Grube). *Doklady Biol. Sci.* **125:** 935–937.

EPSHTEIN, V. M. 1961. A review of the fish leeches (Hirudinea: Piscicolidae) from northern seas of SSSR. *Doklady Biol. Sci.* **141:** 1121–1124.

GIBSON, R. N., AND L. J. TONG. 1969. Observations on the biology of the marine leech *Oceanobdella blennii. J. Mar. Biol. Assoc. U. K.* **49:** 433–438.

HALVORSEN, O. 1971. Studies of the helminth fauna of Norway, XIX. The seasonal cycle and microhabitat preference of the leech, *Cystobranchus mammillatus* (Malm 1863) parasitizing burbot, *Lota lota* (Linnaeus). *Norw. J. Zool.* **19:** 177–180.

HALVORSEN, O. 1972. Studies of the helminth fauna of Norway, XXX. Seasonal cycles of fish parasites in the River Glomma. *Norw. J. Zool.* **20:** 9–18.

HERMAN, S. S. 1963. Vertical migration of the opossum shrimp, *Neomysis americana* (Smith). *Limnol. Oceanogr.* **8:** 228–238.

HESTHAGEN, I. H. 1973. Diurnal and seasonal variations in the near-bottom fauna—the hyperbenthos—in one of the deeper channels of the Kieler Bucht (Western Baltic). *Kiel. Meeresforsch.* **XXIX(2):** 116–140.

HODGE, D. 1963. The distribution and ecology of the mysids in the Brisbane River. *Queensland Univ., Brisbane Dept. Zool., Papers* **2:** 91–104.

HOFFMAN, J. 1955. Quelque characteres ethologiques de la Piscicolidee: *Cystobranchus respirans* Troschel. *Archiv. Inst. Grand-Ducal de Luxembourg.* N. S. **22:** 223–235.

HOLTHUIS, L. B. 1954. Mysidacea. Pp. 213–219 in L. F. de Beaufort, Ed., *Veranderung in de Flora en Fauna der Zuidersee.* Nederlandse dierkundige Vereniging den Helder, Netherlands.

HULBURT, E. M. 1957. The distribution of *Neomysis americana* in the estuary of the Delaware River. *Limnol. Oceanogr.* **2:** 1–11.

KINNE, O. 1955. *Neomysis vulgaris* (Thompson): eine autokologisch-biologische Studie. *Biol. Zentralbl.* **74:** 160–202.

MAUCHLINE, J. 1967. The biology of *Schistomysis spiritus* (Crustacea, Mysidacea). *J. Mar. Biol. Assoc. U. K.* **47:** 383–396.

MEYER, M. C., AND A. A. BARDEN. 1955. Leeches symbiotic on Arthropoda, especially decapod Crustacea. *Wasmann J. Biol.* **13:** 297–311.

MOORE, J. P., AND M. C. MEYER. 1951. Leeches (Hirudinea) of Alaska and adjacent waters. *Wasmann J. Biol.* **9:** 11–77.

Pezzack, D. S., and S. Corey. 1979. The life history and distribution of *Neomysis americana* (Smith) (Crustacea, Mysidacea) in Passamaquoddy Bay. *Can. J. Zool.* **57:** 785–793.

Sandine, P. H., K. A. Tighe, and H. W. Hoffman. 1977. Entrainment. Pp. 3–26 in T. R. Tatham, P. H. Sandine, R. P. Smith, H. W. Hoffman, K. A. Tighe, and D. L. Thomas. *Preliminary Final Report of Ecological Studies for the Oyster Creek Generating Station.* Ichthyological Assoc., Inc., Absecon, New Jersey.

Sawyer, R. T., and D. L. Hammond. 1973. Observations on the marine leech *Calliobdella carolinensis* (Hirudinea: Piscicolidae) epizootic on the Atlantic menhaden. *Biol. Bull.* **145:** 373–388.

Sawyer, R. T., and M. G. White. 1969. A new genus and species of marine leech, *Glyptonotobdella antarctica*, from an Antarctic isopod. *Br. Antarct. Surv. Bull.* **22:** 1–14.

Selensky, W. D. 1923. *Crangonobdella murmanica* n.g., n.sp., ein auf *Sclerocrangon schmarotzende* Ichthyobdellidae. *Zool. Jahrb.* **46:** 397–488.

Selensky, W. D. 1927. On a new Ichthyobdellidae (*Mysidobdella oculata* n.gen. n.sp.) a parasite on Crustacea. *Proc. 2nd Cong. of Zoologists, Anatomists, and Histologists of USSR,* **2:** 32–33. (In Russian).

Tattersall, W. M. 1938. The seasonal occurrence of mysids off Plymouth. *J. Mar. Biol. Assoc. U. K.* **23:** 43–56.

Thompson, D. H. 1927. An epidemic of leeches on fishes in Rock River. *Bull. Ill. State Nat. Hist. Surv.* **17:** 195–201.

Vasileyev, E. A. 1939. The Ichthyobdellidae of the Far East. *Trudy Karel. Gos. Pedagog. Inst., Ser. Biol.* **1:** 25–66.

Vorstman, A. G. 1951. A year's investigation into the life cycle of *Neomysis vulgaris* Thompson. *Verhandlungen der Internationalen Vereinigung fur Theoretische und angewandie Limnologie* **II:** 437–445.

Wigley, R. L., and B. R. Burns. 1971. Distribution and biology of mysids (Crustacea, Mysidacea) from the Atlantic coast of the United States in the NMFS Woods Hole collection. *U. S. Fish. Wildl. Serv. Fish. Bull.* **69:** 717–746.

Zmudzinski, L. 1967. Seasonal migrations of coldwater fauna in the Gdansk Bay. *Cons. Perm. Int. Explor. Mer, 2(2), Ann. Biol.* **21:** 65–67.

Reference: *Biol. Bull.*, **160:** 11–30. (February, 1981)

HISTOLOGICAL AND PHYSIOLOGICAL ASPECTS OF THE MEDULLA EXTERNA X ORGAN, A NEUROSECRETORY CELL GROUP IN THE EYESTALK OF *PALAEMON SERRATUS* PENNANT (CRUSTACEA, DECAPODA, NATANTIA)

CHANTAL BELLON-HUMBERT, FRANÇOIS VAN HERP,[1]
GODFRIED E. C. M. STROLENBERG,[1] AND J. MANUEL DENUCÉ[1]

Laboratoire de Biochimie, Faculté de Médecine, Université de Bretagne Occidentale,
29279 Brest Cédex, France

ABSTRACT

The medulla externa X organ (MEX) is composed of two cell types containing two different categories of neurosecretory elementary granules also found in the sinus gland. Changes in the volume and activity of the organ were observed in relation to the intermolt cycle and season. After extirpation of the medulla externa X organ, the volumes of both the sinus gland and the organ of Bellonci increased in post- and intermolt stages. In the sinus gland, lysis and clumping were observed in axon endings containing one (large) granule type. Also as a consequence of the extirpation, large droplets were secreted in vacuoles of the organ of Bellonci. Neurosecretory cells in the medulla terminalis ganglionaris X organs (MTGX) showed increased activity and hypertrophy. The medulla externa X organ also had an impact on the distal retinal and chromatophoric red pigments.

INTRODUCTION

The eyestalks in decapod crustaceans are complex appendages considered to be the main source of hormones acting in several physiological and developmental processes.

The anatomy and cytology of the nervous and neurosecretory structures of the optic ganglia, well known since Hanström's work (1928, 1947), have been described in numerous light-microscopic investigations. However, few studies deal with changes in the neurosecretory pathways of decapods after surgical interruptions. Several authors have been successful in removing the sinus gland in decapods (Brown, 1942; Scudamore, 1942; Panouse, 1944; Kleinholz, 1947; Stephens, 1951). But only Passano (1951a) and Bliss and Welsh (1952) observed changes so produced in eyestalk structures. Extirpation of the neurosecretory cell centers, carried out by Bliss and Welsh (1952) and by Passano (1953), gave a first idea of the histological changes induced. Since then, no selective surgical experiments on the neurosecretory cell groups in the decapod eyestalk have been reported, although the removal of the organ of Bellonci and the medulla externa X organ (MEX) in the Natantia *Palaemon serratus* is technically possible (Pasteur, 1958).

Our objective was to determine whether the chromatophore index and the blood

Received 15 December 1979, accepted 24 November 1980.

Abbreviations: CHH: Crustacean hyperglycemic hormone; DRPH: Distal retinal pigment hormone; MEX: Medulla externa X organ; MTGX 1 and 2: Medulla terminalis X organs 1 and 2; P.A.F.: Paraldehyde fuchsin; RPCH: Red pigment concentrating hormone.

[1] Zoölogisch Laboratorium, Faculteit der Wiskunde en Natuurwetenschappen, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, Nederland.

sugar content of prawns change after extirpation and injection of the MEX organ. Special attention was paid to the neurosecretory characteristics of the MEX cells, and to exogenous and endogenous factors that eventually modulate these cells. Effects on neurosecretory eyestalk structures remaining after removal of the MEX organ were also studied.

Materials and Methods

Collection and maintenance of specimens

Palaemon serratus Pennant adults were collected during February, April, July, and November of 1977, in the Bay of Concarneau (France). They were maintained in translucent containers with recycled aerated sea water (pH 8.0; 30‰ salinity). Room temperature varied between 17°C in winter and 22°C in summer. The natural photoperiod was season-bound and ranged between 14 h darkness:10 h light in February, 9½ h darkness:14½ h light in April, 8 h darkness:16 h light in July, and 12 h darkness:12 h light in November. The light intensity above the aquaria varied between 800 and 2800 lux in February, 800 and 4000 lux in April, 8000 and 16000 lux in July, and 600 and 3000 lux in November. The animals were fed granulated food, fresh mussels, and fish. For each experiment, prawns were selected according to molting stage, sex, and size.

Procedure for light microscopy

Eyestalks were removed between 3 and 4 p.m. and fixed according to Halmi (Gabe, 1968) for 24 h. After dehydration in 95% ethanol and n-butanol (3 changes for 3 h in each medium), they were embedded in Histomed (58°C melting point). Sections 7 μm thick (transversal and longitudinal) were stained with the azan novum method of Geidies (Gabe, 1968). Masson's and Gabe's trichrome staining methods were also used. Neurosecretory cells were stained with paraldehyde fuchsin, according to Gabe (1953).

Morphometric measurements were carried out with a "Manuell Optisches Bild Analyse System (MOP) Kontron" for determining areas and diameters. Volumes of structures were calculated using the following formula: $(t \times d \times k)/m$, where t = total area, d = thickness of the section, k = order of selection of the sections, and m = magnification, as previously described (Van Herp *et al.*, 1977).

Numerical data were worked out statistically by Student's t test.

Procedure for electron microscopy

For transmission electron microscopy, extirpated organs were immediately fixed in cold 2.5% glutaraldehyde buffered with 0.1 *M* sodium cacodylate, pH 7.3; and postfixed in 1% osmium tetroxide dissolved in the same buffer. After dehydration in an ascending series of ethanol concentrations, the organs were embedded in a mixture based on Epon 812 (sol. A: Epon 812 (62 cc) + dodecenyl succinic anhydride (100 cc); sol. B: Epon 812 (100 cc) + methyl nadic anhydride (89 cc); mixture: 5 cc sol. A + 5 cc sol. B + 0.15 cc DMP_{30}). Silver to gray ultrathin sections were cut with a Reichert O.M.U. 2 Ultramicrotome. After poststaining with uranyl acetate (Watson, 1968) and lead citrate (Reynolds, 1963), the sections were examined with a Philips 300 electron microscope at 60 kV.

For scanning electron microscopy, eyestalks were first washed in 0.1% ammonia in distilled water before fixation in absolute ethanol-chloroform (2:1) for 1 week.

They were rinsed in absolute ethanol and in n-butanol (3 changes in each medium). After gold-palladium coating, samples were examined with a Cambridge S 4 electron microscope. Best resolution was obtained at 20 kV with a current of 150 μA.

Technique of MEX extirpation in living prawns

The prawn was placed on its ventral side into a notch in a cork plate and strongly attached there with a flexible sheet of plastic. Using a dissection microscope (magnification: ×25), the left eye of the prawn was pulled back from the rostrum. Four perforations were made with a sharp stainless steel needle in the rostrodorsal area of the eyestalk below the ommatidia. A cuticular piece (0.25 mm^2) was cut away. The underlying epidermis was then removed with an iridectomy knife. Because of its thickness, the connective tissue sheath covering the MEX organ was carefully extirpated with sharp tweezers, revealing the bluish-white MEX organ attached to the medulla externa. Under slight pressure it was lifted and picked up with tweezers. A superficial cauterization was necessary to limit bleeding and infection.

The whole extirpation required about 8 min. Twenty-four h later, the right eyestalk was cut off and the stub cauterized.

Histological preparations showed that the optic ganglia were not impaired. Healing tissue was found in the operated area. Sham operations showed that ommatidia were not directly damaged by the operation. Normal retinal pigment migrations were observed the first day after the extirpation in all operated animals. They persisted after sham operations, even without cauterization.

All extirpations were carried out during stage C, according to the definition of molt stages by Drach and Tchernigovtzeff (1967). The histological investigations were carried out after the next molt.

Survival was good: mortality did not exceed 5% during the first week, and reached 30% 1 month after the following molt.

Physiological assays

Chromatophore index estimation: After extirpation, the red pigment in the chromatophores was examined daily. The measurements were recorded in Panouse's scale (Panouse, 1946), using six stages to describe pigment dispersion (0: full pigment concentration, rounded chromatophore; 1: irregular shaped chromatophore; 2: stellate chromatophore with some large branches; 3: sea-urchin-like aspect with bifurcated chromorhizae; 4: highly bifurcated and fine chromorhizae, chromatophores still distinct; 5: full dispersion, adjacent chromatophores with intermingled chromorhizae). Measurements were always made in the dorsal area of the first abdominal segment at 10 and 12 a.m., on pools of 10 intact and 10 operated animals. From these values, the mean daily degree of dispersion was calculated, in order to relate pigment movement to time.

The circadian rhythm was studied on groups of 15 intact and 15 operated animals. For this purpose, the pigment index was evaluated every 2 h (daytime) or 3 h (nighttime) on the first and the eighth day after the operation.

Crude MEX extracts were prepared in sea water, centrifuged at 3000 *g* for 20 min and injected into eyestalkless intermolt prawns (0.2 ml/animal). Sixty min were allowed for measurements. Chromatophores were also examined after injection of either boiled or lyophilized extracts.

Glycemia measurements: Blood samples were collected by cutting off the telson.

The samples were pooled, frozen for 48 h, thawed, and centrifuged. Glucose was measured in the serum using the "GOD perid" test kit (Boehringer, Mannheim) and expressed as μg/ml.

In extirpation experiments, blood samples were collected at 12 a.m. from operated animals in the premolt stages (D'_1–D'''_1) and again 12 and 48 h after operation. For injection purposes, the MEX material was homogenized in distilled water at 4°C, extracted for 2 h, and lyophilized. The equivalent of one organ was suspended in 50 μl (⅓ distilled water: ⅔ sea water) and injected into the abdomen of an eyestalkless intermolt prawn. Blood samples were taken 2 h later.

RESULTS

General description of the MEX organ

Morphological observations: The MEX organ is a well defined group of cells located dorsolaterally on the rostral side of the eyestalk and at the surface of the medulla externa, opposite the sinus gland and facing the upper part of the organ of Bellonci, which lies on the ventral side (Figs. 1, 2, 3, 4).

At the base of the organ there seems to be a nervous connection with the ventral region of the sensory pores, near the organ of Bellonci (Fig. 3). In addition, axons from the distal MEX cells joining the lamina ganglionaris were identified microscopically. The relationship with the sinus gland awaits confirmation from iontophoresis experiments: axons, tangential to the medulla externa, pass to the distal

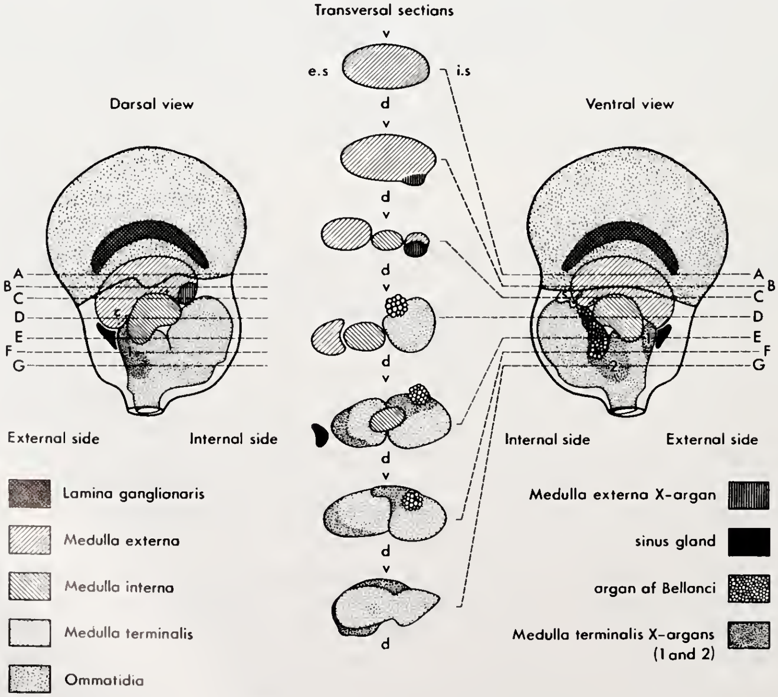

Transversal sections
v
e.s
i.s
d
Dorsal view
Ventral view
A
B
C
D
E
F
G
c
s
1
2
External side
Internal side
Internal side
External side
Lamina ganglionaris
Medulla externa
Medulla interna
Medulla terminalis
Ommatidia
Medulla externa X-organ
sinus gland
organ of Bellonci
Medulla terminalis X-organs (1 and 2)

FIGURE 1. General organization of a left eyestalk of the prawn *Palaemon serratus*: dorsal and ventral views, transverse sections. (d: dorsal side; v: ventral side; e.s.: external side; i.s.: internal side; c (left): scattered neurosecretory cells; s (right): sensory pores; A, B, C, D, E, F, G: transverse sections).

part of the gland. A previous publication (Van Herp *et al.*, 1977) described the light-microscopic characteristics of the cell types in the MEX organ. Five cell types could be distinguished by appropriate staining and measuring the cell bodies and nuclei. Some of these cells are shown in Figure 6.

Electron micrographs show two types of neurosecretory cells in the MEX organ. They differ in elementary granules and the endoplasmic reticulum.

Type 1 cells have electron-dense elementary granules with a mean diameter of 130 nm. They are rather scarce, in spite of the abundant Golgi apparatus and ribosomes. The rough endoplasmic reticulum has well developed lamellae. The smooth endoplasmic reticulum consists of heavily packed tubules. Mitochondria are few. The nuclei are very dense and irregular (Figs. 7, 9).

Type 2 cells contain granules of about 96 nm diameter and much lower electron density. Rough endoplasmic reticulum is abundant throughout the cytoplasm. In contrast to the first cell type, mitochondria are very numerous. Active Golgi complexes showing condensing vacuoles and multivesicular bodies are apparent in some cytoplasmic areas. The round nuclei show areas with very dense chromatin (Figs. 8, 10).

The two cell types appear to have different locations in the organ: the first cell type is more common at the edge of the organ; the second is more abundant in contact with the medulla.

Morphometric observations: To relate activity of the MEX organ to molting cycle, season, sex, and size of animals, the volume of the organ was measured and the number of cells larger than 20 μm was determined (Table I). The organ itself does not seem to change much in volume during different stages of the molting cycle, but a slight yet significant change was observed in the number of active neurosecretory cells: They are more numerous in postmolt and intermolt than in premolt. No significant seasonal influences on the MEX organ could be detected, except for a slight increase in volume during summer. In small prawns the large neurosecretory cells seemed slightly less numerous. No other significant differences due to sex or size were noticed.

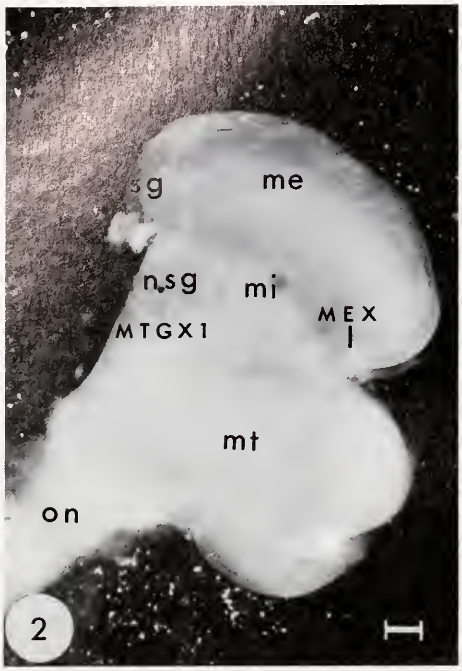
Histophysiological impact of MEX organ extirpation

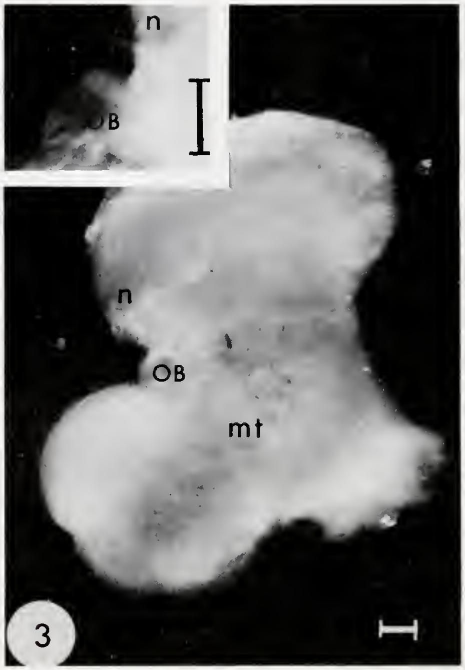
Morphological observations in the eyestalk: The new cuticle showed no trace of the wound after the postmolt, nor was there a gap in the epidermis inside the eyestalk. The optic ganglia appeared normal. Connective tissue and large blood lacunes filled the area of extirpation.

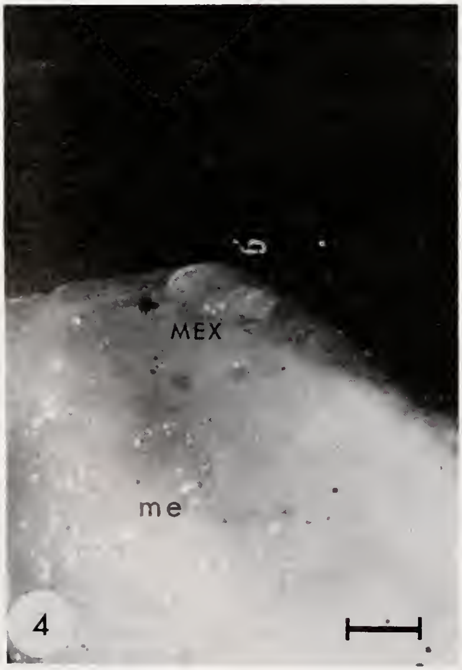
After the extirpation, the sinus gland appeared large and clear, in contrast with its milky appearance in controls. Microscopic examination revealed no important changes, except that orange azan-stained clumps of droplets in the lacunar spaces of the inner blood sinus appeared more numerous, chiefly in stage B.

The possible impact of the operation on the sinus gland was also studied with the electron microscope. A previous publication (Strolenberg *et al.*, 1977) described five granule types with mean diameters of 70, 79, 98, 125, and 150 nm. In the present study, the frequency of granule types smaller than 100 nm in stage C control animals was 57%. Stage C experimental animals had all five types of granules, and about the same frequency distribution (60% granules < 100 nm in diameter). However, granules of 125-nm mean diameter had undergone lysis and clumping (Fig. 11). The outer wall or basement membrane of the gland also seemed thicker after the operation.

As shown in Table II, the sinus gland was twice as large after MEX organ


sg
me
n.sg
mi
MEX
MTGX1
mt
on
2





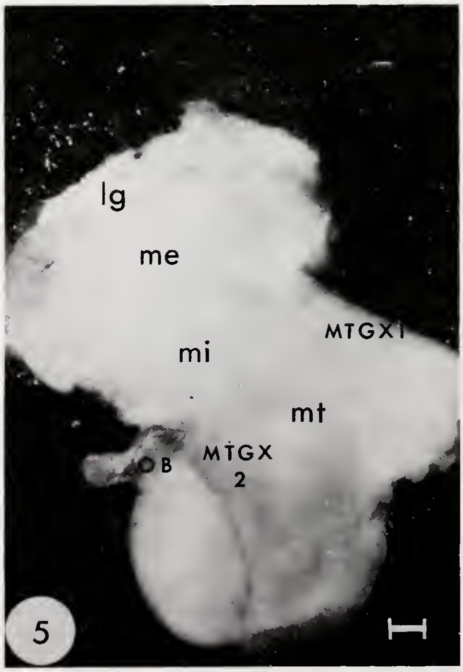

lg
me
mi
MTGX1
mt
OB
MTGX
2
5

FIGURE 2. Dorsal view of a left eyestalk, showing the medullae (me: medulla externa, mi: medulla interna, mt: medulla terminalis), the optical nerve (on), the sinus gland (sg) with its two clearly distinct parts and its nerve (n.sg), and the locations of the MEX and MTGX 1 organs. Scale bar equals 200 μm.

FIGURE 3. Ventral view of a left eyestalk showing the nerve (n) of the medulla externa and the organ of Bellonci (OB), extruding from the medulla terminalis (mt). The inset shows that the nerve does not join the organ of Bellonci. Scale bar equals 200 μm.

extirpation in post- and intermolt animals and comparable in volume to the sinus gland in the premolt stages. The operation had no visible effect on the last stages.

The neurosecretory cells of *Palaemon serratus* are found in the medullae externa and terminalis. In the medulla terminalis they form two groups: the MTGX 1 and MTGX 2 organs. The former is located on the outer side of the ganglia below the sinus gland and the latter lies on the ventral side below the organ of Bellonci (Figs. 1, 2, 5). These groups are characterized by four cell types (Van Herp *et al.*, 1977). Our morphometric studies were concerned with these two organs. A few cells scattered on the ventral side of the medulla externa were not included in the measurements.

Activity of the neurosecretory cells in the MTGX 1 and MTGX 2 organs was measured by counting cells with a diameter larger than 20 μm in control and experimental animals.

Active cells were more numerous during the postmolt and intermolt stages of control and operated animals (Table II). Moreover, MEX extirpation induced cell hypertrophy during these stages: In extirpated animals large cells were significantly more numerous. This hypertrophy was particularly striking in the area of the giant neuron, located in the apical region of the MTGX 1 organ.

Under light microscopy, the lobular structure of the organ of Bellonci in extirpated animals resembles that described for controls. It consists of packs of onion bodies in the distal part and degenerating onion bodies in the proximal part, near the MTGX 2 organ. Onion bodies are typical of the organ of Bellonci: They appear as coiled outer ciliary segments of sensory cells. In these bodies, degenerative processes induce the formation of a central vacuole, filled with an undefined material. In controls, various stages of lysis of onion bodies were observed in these central vacuoles, as shown by droplets of secretion that stained pale blue with azan or light purple with paraldehyde fuchsin (P.A.F.) (Fig. 14). In contrast, in the extirpated animals, the proximal part contained large vacuoles filled with orange azan-stained material, like the aforementioned droplets in the sinus gland. Droplets were also observed outside the organ of Bellonci next to the MTGX 2 organ. These droplets correlated with the molting cycle: They were large during stage B and appeared more granulated in stage C. Only a few were observed in stage D (Figs. 15, 16).

A morphometric estimate of the volume of the entire organ of Bellonci suggests an increase after the first molt in operated animals. This phenomenon became particularly evident during the post- and intermolt stages (Table II).

After the molt following extirpation, there were striking changes in the main and larval sensory pores, located on the rostroventral side of the eyestalk under the ommatidia. In some cases it became impossible with scanning electron microscopy to distinguish the minute double micropores of the main sensory pore and the single micropores of the larval sensory pore. Occasionally, only the large rectangular site of the main sensory pore and the circular impression of the larval sensory pore could be detected (Figs. 12, 13).

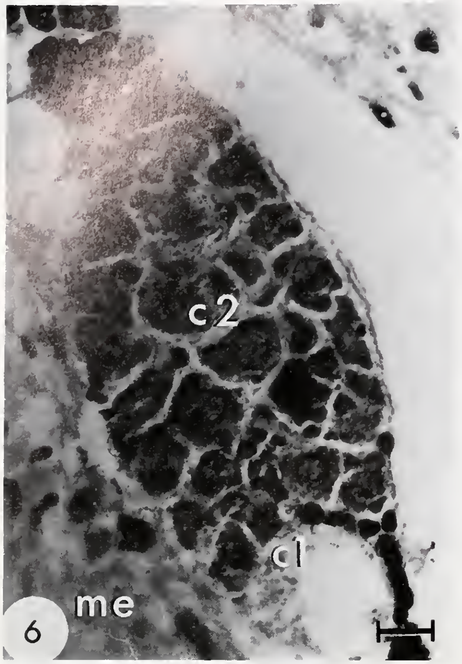
Physiological observations

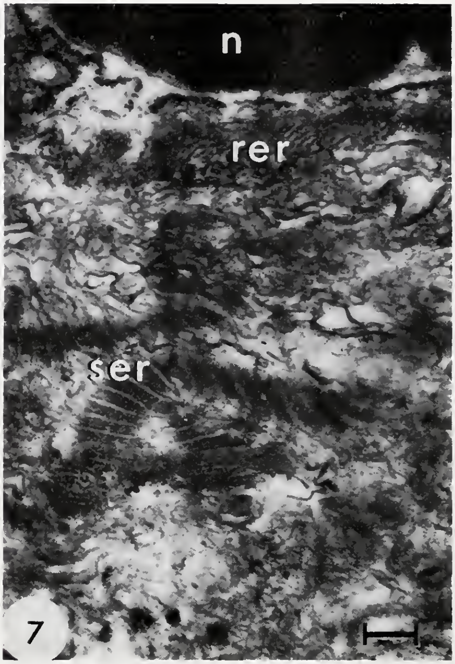
Retinal pigment migration: The day after MEX organ extirpation and before sectioning of the second eyestalk, the dark coloration of the operated eye was

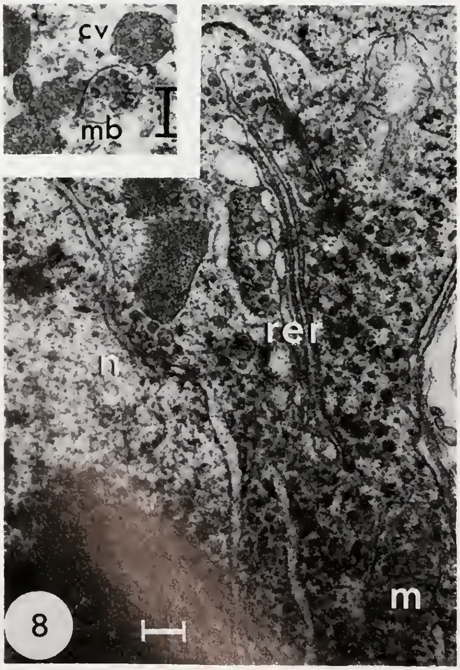
---

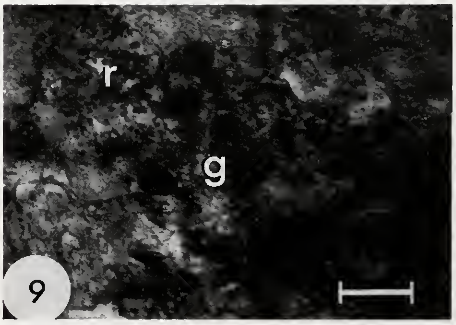
FIGURE 4. View of the MEX organ at the edge of the medulla externa (me). Scale bar equals 200 μm.

FIGURE 5. Ventral view of a left eyestalk, showing the lamina ganglionaris (lg) and the medullae (me, mi, mt), the prominent organ of Bellonci (OB), and the locations of the MTGX organs 1 and 2. The MTGX 1 lies on the external side, under the sinus gland. The MTGX 2 is on the ventral side in contact with the organ of Bellonci. Scale bar equals 200 μm.









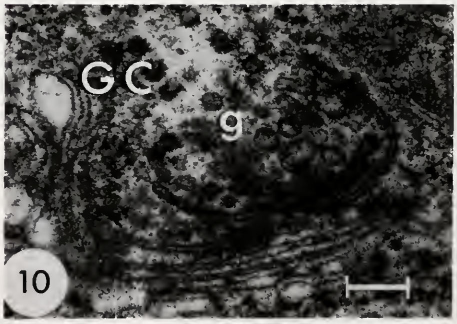


FIGURE 6. Longitudinal section of the MEX organ in light microscopy, showing the neurosecretory cell group (lying against the medulla externa (me)) with its two types of cells: C 1, with dark and irregular nuclei; C 2, with clear and round nuclei. Scale bar equals 25 μm.

FIGURE 7. Ultra-thin section of type 1 neurosecretory cell, with nucleus (n), rough (rer) and smooth (ser) endoplasmic reticulum. Scale bar equals 0.25 μm.

TABLE I

Variations in the MEX organ according to intermolt cycle, season, sex, and size. Level of significance: $p > 0.1$ *= not significant (n.s.);* $0.05 < p < 0.1$ *= barely significant (±s.);* $0.01 < p < 0.02$ *= significant (s.);* $0.001 < p < 0.01$ *= highly significant (h.s.).*

Parameters X = ±SEM	Volume of MEX (mm^3) ± SD		Number of cells >20 μm in MEX ± SD	
Intermolt cycle				
Postmolt (A–B)	0.29 ± 0.04	N = 5	46 ± 7	N = 5
Intermolt (C)	0.28 ± 0.03	N = 2	45 ± 11	N = 2
Premolt (D°–D'''_{1})	0.30 ± 0.04	N = 10	33 ± 3	N = 10
Student's *t* test between different molting stages	all correlations n.s.		A–B/D°–D'''_{1} ± s. A–B–C/D°–D'''_{1} other correlations n.s.	
Seasons				
Feb. 13, 1977	0.22 ± 0.01	N = 2	35 ± 6	N = 2
March 26, 1977	0.27 ± 0.03	N = 11	37 ± 4	N = 11
July 20, 1977	0.37 ± 0.04	N = 3	46 ± 9	N = 3
Nov. 11, 1977	0.43	N = 1	42	N = 1
Student's *t* test between seasons	February/July ± s. other correlations n.s.		all correlations n.s.	
Sex				
Males	0.29 ± 0.04	N = 8	43 ± 4	N = 8
Females	0.31 ± 0.04	N = 8	36 ± 5	N = 8
Student's *t* test between sexes	n.s.		n.s.	
Size				
≤50 mm	0.28 ± 0.04	N = 6	31 ± 2	N = 6
50<, <60 mm	0.27 ± 0.03	N = 6	44 ± 5	N = 6
≥60 mm	0.33 ± 0.07	N = 5	41 ± 5	N = 5
Student's *t* test between different sizes	all correlations n.s.		$T \leq 50$ mm/$50 < T < 60$ mm ± s. other correlations n.s.	

striking when compared to the clear intact eye. In fact, the distal retinal pigment, which usually migrates towards the periphery before darkness, continued to display its night adaptation and never moved during the period of observation. This provided a criterion for a successful operation. This effect was not noticeable after an incomplete MEX organ extirpation, nor after a sham operation or sinus gland extirpation. Its absence after sham operations shows that the effect was not caused by cauterization or bleeding.

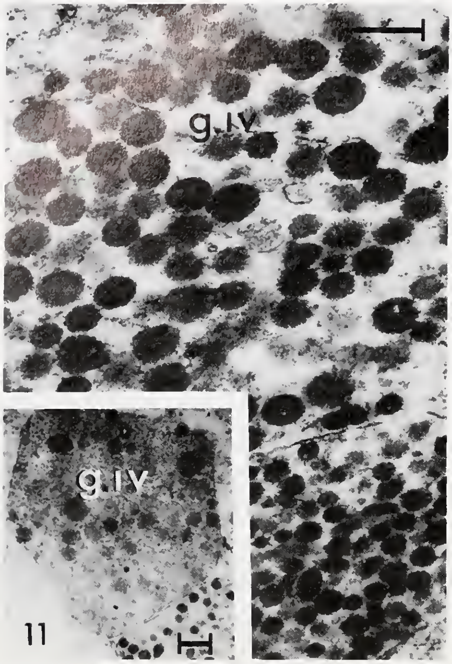
Chromatophore red pigment migration: Three types of chromatophores in the

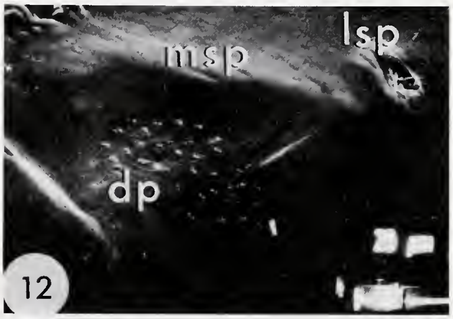
---

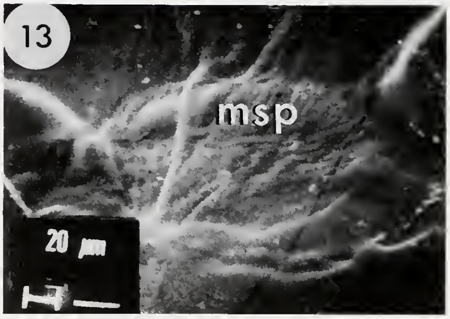
FIGURE 8. Ultra-thin section of a type 2 neurosecretory cell, with nucleus (n), rough endoplasmic reticulum (rer) and mitochondria (m). In the inset, an example of multivesicular body (mb) and condensing vacuole (cv). Scale bars equal 0.25 μm.

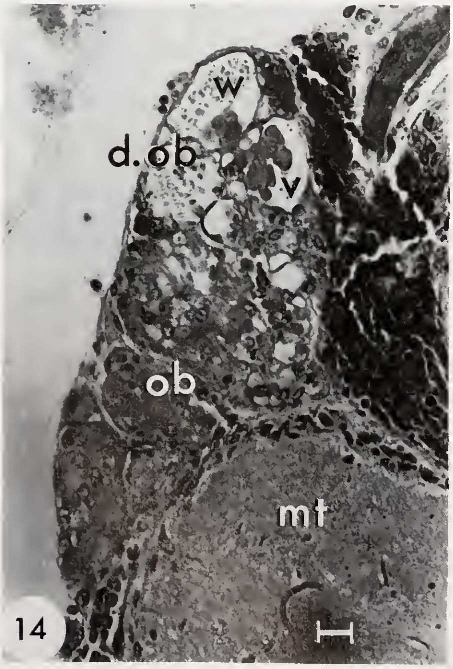
FIGURE 9. Granules (g) of 130-nm diameter (d), from cell type 1. Note their high electron density and the abundance of ribosomes (r). Scale bar equals 0.25 μm.

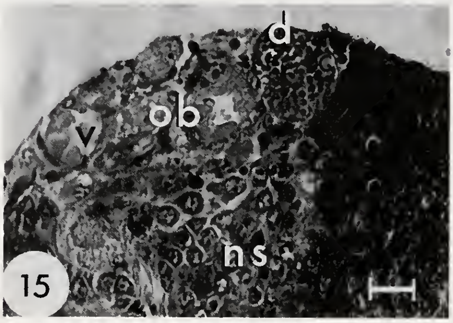
FIGURE 10. Granules (g) of 96-nm diameter, from cell type 2. In this cell type, Golgi cisternae (GC) are well developed. Scale bar equals 0.25 μm.


11


12


13


14


15

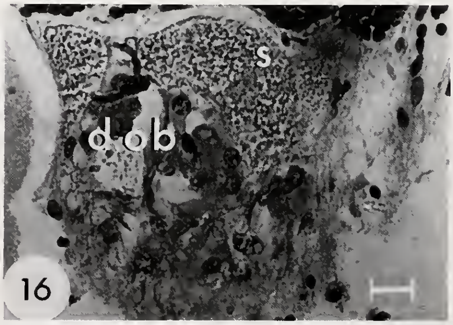

16

FIGURE 11. Ultra-thin section of the sinus gland, showing an axon terminal with granules of 125-nm diameter (g IV) in a control animal. Lysis affects this type of granule after MEX extirpation, as shown in the inset. Scale bars equal 0.25 μm.

FIGURE 12. SEM view of the sensory pores in a control animal (stage C) showing the main sensory pore (msp) with its double micropores (dp) and the larval sensory pore (lsp).

TABLE II

Impact of MEX organ extirpation on the eyestalk structures during the intermolt cycle following the operation. Level of significance: See Table I.

Parameters	A + B + C	D°–D‴	A–D‴	Student's *t* test intermolt cycle
	Volume of the sinus gland (mm^3) ± SD			
Controls	0.27 ± 0.03 N = 7	0.55 ± 0.11 N = 5	—	s.
Operated	0.55 ± 0.10 N = 6	0.62 ± 0.22 N = 4	0.58 ± 0.10 N = 10	n.s.
Student's *t* test operation	s.	n.s.	—	
	Number of cells > 20 μm in MTGX organs 1 and 2 (±SD)			
Controls	33 ± 2 N = 3	8 ± 1 N = 3	—	h.s.
Operated	54 ± 4 N = 4	15 ± 7 N = 2	—	h.s.
Student's *t* test operation	s.	n.s.	—	
	Volume of the organ of Bellonci (mm^3) ± SD			
Controls	1.21 ± 0.01 N = 2	1.38 ± 0.19 N = 7	1.23 ± 0.10 N = 9	n.s.
Operated	2.01 ± 0.28 N = 6	1.47 ± 0.12 N = 2	1.88 ± 0.2 N = 8	n.s.
Student's *t* test operation	± s.	n.s.	h.s.	

hypodermis of *Palaemon serratus* contain red pigment. One type forms stripes in a specific pattern on the body. The second is composed of smaller units scattered in the intervals between stripes. In both instances a yellow pigment is also present. The third type is a large chromatophore which also contains a white pigment. Its location does not vary and its behavior seems more complex. This type of chromatophore is not included in the present study.

FIGURE 13. A comparable view of the main sensory pore (msp) in a MEX extirpated animal in postmolt following the operation. The aperture of micropores is no longer visible.

FIGURE 14. Light-microscopy of a transverse section of the organ of Bellonci in a control animal, at the level of medulla terminalis (mt), with packs of onion bodies (ob), degenerating onion bodies (d.ob) and their waste (w) filling vacuoles (v). Scale bar equals 25 μm.

FIGURE 15. Section in light microscopy of the organ of Bellonci in a MEX extirpated prawn (stage B), showing onion bodies (ob) and large droplets (d) filling spaces in the organ. Note also the presence of neurosecretory cells (ns) of MTGX 2 in close contact with the organ of Bellonci. Scale bar equals 25 μm.

FIGURE 16. Section in light microscopy of the organ of Bellonci in a MEX extirpated prawn (stage C), showing the degenerating onion bodies (d.ob). A thin granular secretion (s) here takes the place of droplets. Scale bar equals 25 μm.

After extirpation of the MEX organ and sectioning of the remaining eyestalk, the red pigment in the chromatophores began to expand. Pigment dispersion increased for several days (Fig. 17). The speed of the reaction was linked to several factors, such as temperature, photoperiodicity, and molting stage. The chromatophores also displayed diurnal pulsations.

As a rule, operated animals became reddish brown after the molt following the operation, but a few animals remained pale. The eyestalks of these prawns invariably displayed a few non-extirpated MEX cells or some large neurosecretory cells on the ventral side of the medulla externa. In general, their MTGX organs appeared more developed.

Compared with MEX organ extirpation, sinusglandectomy immediately induced a strong dispersion of the red pigment which persisted for only a short while. Chromatophores contracted progressively from the second or third day after the operation.

As shown in Figure 18, the circadian rhythm in control animals was biphasic: on a white or sandy background, the chromatophores, especially on stripes, were contracted during the night and more or less dispersed during the day. In operated animals this rhythm was lost, the loss being related to an increased dispersion; and on a white background, the rhythm tended to be reversed. Pigment was concentrated early in the morning and dispersed before sunset. Operated animals became more colored during the night than during the day. These responses could be detected when the animals turned darker, a few days after the operation.

Injections of crude MEX-organ extracts produced concentration of the red pigment in the dispersed chromatophores of eyestalkless animals. The strength and duration of the response were related to the concentration of the extracts, but the speed of the response did not change. Full contraction could be detected 15/20 min after the injection. The reaction after injections of boiled MEX extracts was stronger, and the chromatophoric responses induced by lyophilized extracts were comparable to those induced by sinus gland or total eyestalk extracts (Fig. 19).

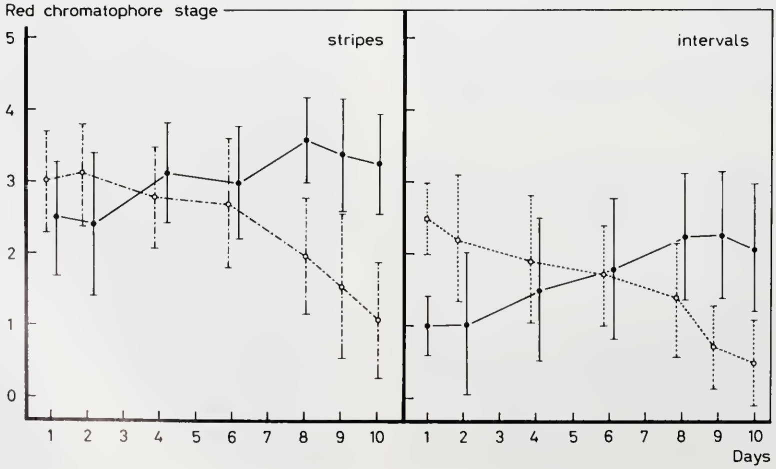


FIGURE 17. Mean dispersion, determined daily, of red chromatophores on stripes and intervals between stripes in experimental prawns kept on a white background. Solid lines: MEX extirpated prawns, dashed and dotted lines: sinus-glandless prawns. Each point represents mean of 10 animals. Chromatophore stages refer to Panouse's scale (see in text: procedure for physiological assays). Vertical bars represent ± one standard deviation (SD) from the mean.

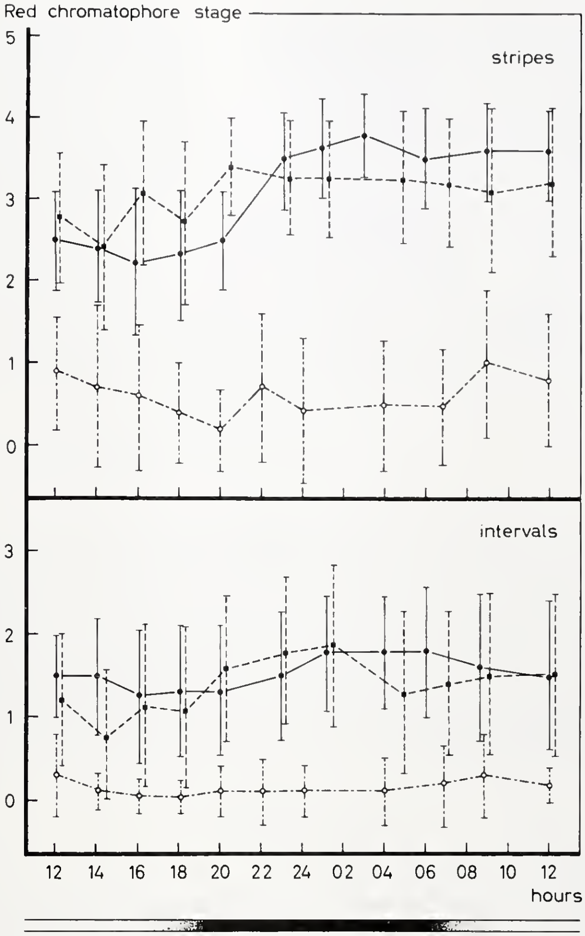


FIGURE 18. Circadian rhythm in red chromatophores (on stripes and in the intervals between stripes) of MEX extirpated and control prawns, kept on a white background. Controls were observed on the first day (dashed and dotted lines), MEX extirpated animals on the first (solid lines) and eighth (dashed lines) days. Each point represents mean of 15 animals, observed in March. Vertical bars: ± 1 SD.

Glycemia determination: In a number of decapod Crustacea, blood glucose level is controlled by a hyperglycemic hormone (CHH) released by the sinus gland. In *Palaemon serratus* the neurosecretory cells that may be the source of this hormone have not been identified. To test the role of the MEX neurosecretory cell group in blood sugar regulation, lyophilized MEX or sinus gland extracts were injected into eyestalkless intermolt prawns. The glucose concentration in the hemolymph showed a 15-fold increase after sinus gland injection (232.5 ± 97.5 μg/ml versus 17.5 ± 2.0 μg/ml in control animals injected with sea water). Injection of lyophilized MEX organs caused a less dramatic rise in blood glucose (33.5 ± 18.5 μg/ml).

The influence of MEX extirpation was studied in premolt prawns, which normally have a high blood glucose level (30 to 40 g/ml). After MEX organ removal, the level was still this high 12 h after the operation, but by 2 days after MEX organ removal it had decreased sharply (17 μg/ml).

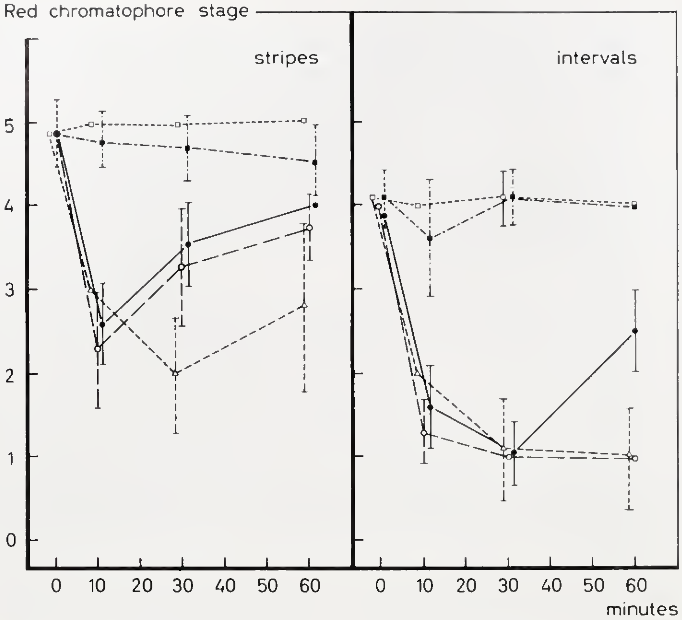


FIGURE 19. Effect of injected lyophilized extracts of eyestalks (shorter-dashed lines), MEX organs (solid lines), and sinus glands (longer-dashed lines) on dispersed red chromatophores (stripes and intervals) of eyestalkless prawns. Injections of muscle extracts (dashed and dotted lines) and sea water (dotted lines) were compared. Each point represents the mean of 10 animals; vertical bars: ± 1 SD. Injection: 1 structure equivalent/animal.

DISCUSSION

General description of the MEX organ

Like the MTGX organs, the MEX organ appears distinctly at metamorphosis (Bellon-Humbert *et al.*, 1978). Most neurosecretory cells in the medulla externa form a well defined group in Natantia such as *Pandalus borealis* (Carlisle, 1959), *Palaemon serratus* (Humbert, 1965; Van Herp *et al.*, 1977), *Palaemon paucidens* (Hisano, 1974, 1976), and *Penaeus japonicus* (Nakamura, 1974).

However, in other Natantia, such as *Typhlatya garciai* (Juberthie-Jupeau, 1976) and *Atyaephyra desmaresti* (Boissou *et al.*, 1976), the neurosecretory cells are more or less scattered in the medulla externa. In other Crustacea, such as the Brachyura (Enami, 1951; Bliss and Welsh, 1952; Matsumoto, 1958; Smith and Naylor, 1972) or Anomura (Farges, 1975a; Bursey, 1975), the MEX organ is more compact. In Macrura, only scattered neurosecretory cells were described in the medulla externa (Durand, 1956; Andrew *et al.*, 1978; Shivers, 1967; Van Herp, 1972).

In Crustacea lacking a distinct MEX organ, the neurosecretory cells are mainly in two locations: above and on the opposite side of the sinus gland. In a few Natantia, neurosecretory cells are in the medulla externa near the sinus gland (Boissou *et al.*, 1976). They can aggregate to form a distinct organ, as in *Pandalus borealis* (Carlisle, 1959). In *Palaemon serratus*, the MEX organ is localized on the rostrodorsal edge of the medulla externa, at the junction with the medulla interna. Only a restricted number of neurosecretory cells are visible on its ventral side, near the distal part of the organ of Bellonci.

Light microscopy showed two to four cell types in the MEX organ. Their neurosecretory nature was established after appropriate staining, *e.g.* with P.A.F. Some small cells also were considered neurosecretory. However, some "stained neurosecretory bodies" may be lysosomes, as mentioned by Hisano (1976) and Andrew *et al.* (1978). One large and one small cell type can always be distinguished in the MEX organ (Miyawaki, 1956; Matsumoto, 1958; Lake, 1970; Hisano, 1974). Our ultrastructural results demonstrating two cell types with two different types of granules (diameters 96 and 130 nm) corroborate this view. A striking similarity exists between these two granule types and those (98 and 125 nm) of the sinus gland of the same species (Strolenberg *et al.*, 1977).

On the basis of histological observations of the MEX organ, we suspect a relationship between volume and number of active cells and the intermolt cycle, the size of the animals, and the season. In living prawns used for experiments, these variations were also noticeable in changes in organ color and size.

Histophysiological influence of MEX organ extirpation

An increase in the volume of the sinus gland and of the organ of Bellonci, as well as hypertrophy of the neurosecretory cells in the MTGX organs, became noticeable in stages A, B, and C after the molt following the operation.

In control animals, the volume of the sinus gland increased during premolt and decreased during postmolt. After MEX extirpation, the premolt aspect of the gland persisted, even after the molt ended; operated animals' glands then appeared twice as large as in controls kept in the same conditions.

Pyle (1943) found large quantities of acidophilic secretions in the sinus glands of premolt *Cambarus affinis* and *Homarus americanus*. This material decreased sharply after ecdysis. Gabe (1952) analyzed the sinus glands of 15 species of decapod Crustacea, including the prawns *Crangon vulgaris, Athanas nitescens*, and *Palaemon serratus*. In all species one type of secretion was more abundant during premolt than in post- or intermolt. Martin (1972), working on the oniscoid *Porcellio dilatatus*, noticed that secretory material began to fill up the sinus gland in stage C and reached a maximum in stage D. Farges (1975a) found the sinus gland in Diogenidae loaded with secretory substances in stage C_4 but empty in other stages. Armstrong (1973) observed also in the shrimp *Palaemon kadiakensis* variations in granule types correlated with the intermolt cycle. In *Astacus leptodactylus* Strolenberg (1979) observed a thickening of the basement membrane during premolt and assumed that this might act as a barrier to prevent the release of neurosecretory granules. Our own observations in extirpated prawns seem to support this assumption.

Lysis of granules with mean diameter 125 nm was observed in axon terminals. A similar phenomenon was reported by Martin (1972) in the sinus gland of *Porcellio dilatatus* after the extirpation of the median region of the protocerebron.

From these observations, it appears that this granule type originates in the MEX organ.

Some changes in the organ of Bellonci have been reported in relation to season (Carlisle, 1959) and molting stage (Drach and Gabe, 1963; Daguerre de Hureaux, 1967; Kauri, 1976). It is generally believed that the role of the organ of Bellonci is not exclusively sensory.

The increase in volume of the organ of Bellonci in the operated animals was related to the presence of droplets, staining orange with azan, between the perineural tissue and the degenerated onion bodies. A comparable secretion was noticed in *Pandalus borealis* by Carlisle (1959) in the organ of Bellonci of females during vitellogenesis and in males approaching sex reversal. Drach and Gabe (1963) described in *Squilla mantis* the elaboration of acidophilic droplets from vacuolated onion bodies, reaching a peak during postmolt (B_2). This was afterwards confirmed by electron microscopic observations (Jacques, 1969; Jacques and Chaigneau, 1972). Farges (1975b) observed in Diogenidae different stages of degeneration of onion bodies and formation of large clumps ("mottes"). Using electron microscopy, Chaigneau (1976, 1977) described dense clumps of a few microns in the supporting cells, and interpreted them as remnants of degenerated onion bodies.

The production of granules by supporting cells was also suggested by several light microscopic and ultrastructural studies (Lake and Ong, 1972; Kauri and Lake, 1972; Dahl and Kauri, 1976; Chaigneau and Juchault, 1974).

Cyclic variations in the activity of the neurosecretory cells of the medulla terminalis have been linked with the intermolt cycle. Thus Drach and Gabe (1962) distinguished two maxima and two minima in the activity of the MTGX organs of *Palaemon serratus*. Our morphometric analysis is in agreement with these data.

Extirpations of the sinus gland by Passano (1951b) in several species of Brachyura and by Bliss and Welsh (1952) in *Gecarcinus lateralis* were followed by extensive storage of neurosecretory material in the vicinity of the MTGX. In some cases, the activity of these neurosecretory cells was so high that the accumulated material was referred to as a "false sinus gland" (Passano, 1951a).

Our experimental data indicate that removing one neurosecretory center, in this case the MEX organ, induces hypertrophy in other neurosecretory cell groups, such as the MTGX organs.

It is difficult to interpret the striking changes in the aspect of the main and larval sensory pores after MEX extirpation. Little is known about innervation and cyclic variations of the sensory pore X organ. Farges (1975b) described in Diogenidae a very important connection between the sensory pore and a neurosecretory cell group located at the junction of the medullae externa and interna. In the eyestalk of *Astacus leptodactylus*, there is a nervous connection between a sensory formation and the medulla interna during larval and adult life (Van Herp *et al.*, 1979). The changes observed in the sensory complex after MEX extirpation may have been induced by interruption of some connection between these structures or by other alterations of the intercellular relations.

Physiological effects

Humbert (1965) reported the influence of the pars distalis of the X organ on chromatophores. She repeated these experiments on the same species but from a different geographic population. Evidently, extirpation of the organ of Bellonci by a dorsal approach (between the medullae and the lobe of the medulla terminalis) also included the MEX organ, while a ventral approach affected a portion of the MTGX 2 organ. It now appears from our selective removal of the MEX organ that the role ascribed to the organ of Bellonci in pigment physiology should be attributed to the MEX organ, removed in the same extirpation.

The MEX organ is involved in control of red pigment. The way it acts on red pigment concentration can be compared to the role of the sinus gland. If red pigment concentrating hormone (RPCH) is carried by the 125 nm granules, then lysis of this granule type after MEX organ extirpation could explain the progressive darkening of the operated animals. But other possibilities exist. According to Shivers (1967), small granules may contain chromatophoric hormones. We found such granules (96 nm) in the MEX organ and the sinus gland. In the latter they were apparently not affected by removal of the MEX organ. If Shivers' hypothesis is correct, the apparent thickening of the basement membrane observed in the premolt sinus gland could delay the release of granules. Perhaps these granules are partially produced by other neurosecretory cells. Our injection experiments also provide evidence for RPCH in the MEX organ. However, the concomitant role of the distal retinal pigments must be elucidated. The nighttime dispersion of distal retinal pigment after the operation could be induced by the lack of DRPH in the sinus gland. It could also result from a change in the release of this hormone. A change in the blood stream, as suggested by Kleinholz (personal communication) is also a possible cause. However, with the same conditions of extirpation and healing, neither sinusglandectomy nor sham operations generated this effect. Though the ommatidia did not seem affected on the days following extirpation, ultrastructural changes induced in the retina by a long-lasting dark adaptation are possible, as reported by Eguchi and Waterman (1979) in *Procambarus clarkii.*

Alterations in retinal pigment behavior might elicit changes in the release of chromatophorotrophins and in chromatophore behavior. This would explain rhythm reversal in operated animals. In these animals, the persistence of a rhythm in the erythrophore response may result from direct action of light on the chromatophores, in the absence of the chief hormonal control, or from the production of RPCH according to a different rhythm by other neurosecretory cell groups (MTGX organs, brain, postcommissural organs).

By applying disc electrophoresis to sinus gland extracts from *Orconectes limosus, Pacifastacus leniusculus,* and *Carcinus maenas*, Keller (1977) found that the CHH material accumulated in the sinus gland is at least 10% of total sinus gland proteins. In comparison, no CHH activity could be detected in electropherograms of medulla terminalis extracts. We were able to provoke hyperglycemia in *Palaemon serratus* by injecting crude sinus gland extracts, but not by injecting MEX material. From this we conclude that CHH is in the sinus gland, but not in the MEX organ. As demonstrated by Van Wormhoudt *et al.* (1978) in a study of the hyperglycemic activity of various endocrine structures in *Palaemon serratus*, a hyperglycemic effect can be obtained after injection of MTGX (1 and 2) material. We therefore suggest that hyperglycemic hormone is produced, not in the MEX organ, but in the MTGX organs, and that it is stored in the sinus gland. CHH-containing cells have been found in the most distal portion of the X organ in the medulla terminalis of *Astacus leptodactylus* (Van Herp and Van Buggenum, 1979), and *Carcinus maenas* (Jaros and Keller, 1979) by an immunocytochemical procedure.

In numerous decapod Crustacea, glucose levels in the hemolymph decline after epedunculation. Moreover, Hamann (1974) described a lower glucose level in *Orconectes limosus* after selective extirpation of the sinus gland. We cannot explain satisfactorily the lower glucose concentration in the blood of *Palaemon serratus* 48 h after MEX extirpation. One possibility is a nonspecific effect induced by extirpation of an arbitrary center in the eyestalk. But the nighttime migration of the distal retinal pigment may be responsible indirectly for a lower glucose level. A circadian rhythmicity in the regulation of the glucose level has been demonstrated by Hamann (1974) for *Orconectes limosus* and by Strolenberg *et al.* (1978) for *Astacus leptodactylus.*

ACKNOWLEDGMENTS

The authors are greatly indebted to Professor H. J. Ceccaldi and Professor H. H. Floch for their stimulating interest, to Miss E. A. J. Derksen for typing the manuscript, to Mr. A. J. M. Coenen for his assistance in the morphometrical approach, to Mr. J. F. Butor for his assistance during the SEM observations, and to Mr. J. Gerritsen for the illustrative work.

This investigation was partially sponsored by C.N.R.S. grant A.T.P. Internationale 1976–2602.

LITERATURE CITED

ANDREW, R. D., I. ORCHARD, AND A. S. M. SALEUDDIN. 1978. Structural reevaluation of the neurosecretory system in the crayfish eyestalk. *Cell Tiss. Res.* **190:** 235–246.

ARMSTRONG, P. V. 1973. Ultrastructure of the sinus gland during the moult cycle of the shrimp *Palaemonetes kadiakensis* (Rathbun). *Am. Zool.* **13:** 1339.

BELLON-HUMBERT, C., M. J. P. THIJSSEN, AND F. VAN HERP. 1978. Development, location and relocation of sensory and neurosecretory sites in the eyestalks during the larval and postlarval life of *Palaemon serratus* (Pennant). *J. Mar. Biol. Assoc. U. K.* **58:** 851–868.

BLISS, D. E., AND J. H. WELSH. 1952. The neurosecretory system of the brachyuran Crustacea. *Biol. Bull.* **103:** 157–169.

BOISSOU, F., D. HUGUET, AND M. VINCENT. 1976. Note préliminaire sur la répartition des cellules neurosécrétrices céphaliques de la crevette d'eau douce *Atyaephyra desmaresti* Millet (Crustacea, Decapoda, Natantia, Atyidae). *Bull. Soc. Sci. Nat. Ouest Fr.* **74:** 91–100.

BROWN, F. A. 1942. Sinus gland extirpation in the crayfish without eyestalk removal. *Proc. Soc. Exp. Biol. Med.* **50:** 295–297.

BURSEY, R. 1975. The microanatomy of the optic ganglia of *Munida irrasa* (Decapoda Galatheidae). *Can. J. Zool.* **54:** 1242–1255.

CARLISLE, D. B. 1959. On the sexual biology of *Pandalus borealis* (Crustacea Decapoda). I. Histology of incretory elements. *J. Mar. Biol. Assoc. U. K.* **38:** 381–394.

CHAIGNEAU, J. 1976. Etude ultrastructurale de l'organe céphalique de Bellonci de Crustacés supérieurs ainsi que du pore sensoriel qui lui est parfois associé. Expérimentation et considérations sur la fonction de ces organes. *Thesis Université de Poitiers.* C. N. R. S. France No. AO 12263. 365 pp., 85 pl.

CHAIGNEAU, J. 1977. L'organe de Bellonci des Crustacés. Mise au point sur l'ultrastructure et sur l'homologie des types avec et sans corps en oignon. *Ann. Sci. Nat. Zool. Biol. Anim.* **19:** 401–438.

CHAIGNEAU, J., AND P. JUCHAULT. 1974. Evolution ultrastructurale, en relation avec l'état sexuel, de l'organe de Bellonci, autochtone et implanté, chez le Crustacé Isopode hermaphrodite *Anilocra frontalis. C. R. Hebd. Séances Acad. Sci. Sér. D Sci. Nat., Paris* **279:** 771–774.

DAGUERRE DE HUREAUX, N. 1967. Etude expérimentale du rôle de l'organe de Bellonci et du lobe optique sur le comportement chromatique et la mue de *Sphaeroma serratum.* Incidence des ablations sur le comportement sexuel. *Bull. Soc. Sci. Nat. Phys. Maroc* **47:** 33–115.

DAHL, E., AND T. KAURI. 1976. Observations on the fine structure of the organ of Bellonci (SPX) in *Halice abyssi* Boeck (Crustacea Peracarida Amphipoda). Chapter VI: pp. 1–15, in T. Kauri, Thesis, University of Lund, Sweden.

DRACH, P., AND M. GABE. 1962. Evolution cyclique de la neurosécrétion de l'organe X des Carididés au cours de l'intermue. *C. R. Hebd. Séances Acad. Sci., Paris.* **254:** 165–167.

DRACH, P., AND M. GABE. 1963. Glandes endocrines protocéphaliques et cycle d'intermue chez *Squilla mantis* Latreille. P. 120 in J. A. Moore, Ed., *Proc. XVIth Congr. Zool.* Washington, D. C.

DRACH, P., AND C. TCHERNIGOVTZEFF. 1967. Sur la méthode de détermination des stades d'intermue et son application générale aux Crustacés. *Vie et Milieu, Sér. A* **18:** 595–610.

DURAND, J. B. 1956. Neurosecretory cell types and their neurosecretory activities in the crayfish. *Biol. Bull.* **111:** 62–76.

EGUCHI, E., AND T. H. WATERMAN. 1979. Long term dark induced fine structural changes in the crayfish photoreceptor membrane. *J. Comp. Physiol.* **131:** 191–203.

ENAMI, M. 1951. The sources and activities of two chromatophorotropic hormones in crabs of the genus *Sesarma.* II. Histology of incretory elements. *Biol. Bull.* **101:** 241–258.

FARGES, G. 1975a. Anatomie comparée du système neurosécréteur protocérébral chez cinq Anomoures. *Bull. Soc. Zool. Fr.* **100:** 477–487.

FARGES, G. 1975b. Etude de l'organe de Bellonci chez les Anomoures. *Bull. Soc. Zool. Fr.* **100:** 489–503.

GABE, M. 1952. Paticularités histochimiques de l'organe de Hanström (organe X) et de la glande du sinus chez quelques crustacés décapodes. *C. R. Hebd. Séances Acad. Sci. Paris* **235:** 90–92.

GABE, M. 1953. Sur quelques applications de la coloration par la fuchsine paraldéhyde. *Bulletin de Microscopie Appliquée* **3:** 153–162.

GABE, M. 1968. *Techniques Histologiques.* Masson Ed., Paris, 1113 pp.

HAMANN, A. 1974. Die neuroendokrine Steuerung tagesrhythmischer Blutzuckerschwenkungen durch die Sinusdrüse beim Flusskrebs. *J. Comp. Physiol.* **89:** 197–214.

HANSTRÖM, B. 1928. *Vergleichende Anatomie des Nervensystems der wirbellosen Tiere.* Springer Verlag, Berlin. 628 pp.

HANSTRÖM, B. 1947. The brain, the sense organs and the incretory organs of the head in the Crustacea Malacostraca. *Kungl. Fysiografiska Sällskapets Handlingar. N. F. Lund,* 58, nr. **9:** 3–44.

HISANO, S. 1974. The eyestalk neurosecretory cell types in the freshwater prawn *Palaemon paucidens.* A light microscopical study. *J. Fac. Sci. Hokkaido Univ. Sér. VI Zool.* **19:** 503–514.

HISANO, S. 1976. Neurosecretory cell types in the eyestalk of the freshwater prawn *Palaemon paucidens.* An electron microscopic study. *Cell Tiss. Res.* **166:** 511–520.

HUMBERT, C. 1965. Etude expérimentale du rôle de l'organe X (pars distalis) dans les changements de couleur et la mue de la crevette *Palaemon serratus. Trav. Inst. Sci. Cherifien, Sér. Zool.* No. 32. 86 pp. 3 pl.

JACQUES, F. 1969. Existence d'un organe sensoriel dans le pédoncule oculaire des larves de Stomatopodes. *C. R. Hebd. Séances Acad. Sci. Sér. D. Sci. Nat. Paris* **268:** 89–90.

JACQUES, F., AND J. CHAIGNEAU. 1972. Observation ultrastructurale de l'organe de Bellonci de la larve de *Squilla mantis* (Crustacé Décapode). *C. R. Hebd. Séances Acad. Sci. Sér. D Sci. Nat. Paris* **274:** 1697–1700.

JAROS, P. P., AND R. KELLER. 1979. Immunocytochemical identification of hyperglycemic hormone-producing cells in the eyestalk of *Carcinus maenas. Cell Tiss. Res.* **204:** 379–385.

JUBERTHIE-JUPEAU, L. 1976. Sur le système neurosécréteur du pédoncule oculaire d'un Décapode souterrain microphtalme *Typhlatya garciai* Chace. *Ann. Spéléol.* **31:** 107–114.

KAURI, T. 1976. The fine structure of the organ of Bellonci in the Crustacea maxillipoda especially *Balanus improvisus* Darwin (Maxillipoda Cirripedia) and *Derocheilocaris remanei* Delamare et Chappuis (Maxillipoda Mystacocarida). Thesis, University of Lund, Sweden. 75 pp.

KAURI, T., AND P. S. LAKE. 1972. The structure of the organ of Bellonci of the syncarid Crustacean *Anaspides tasmaniae* (Thomson). *Z. Zellforsch. Mikrosk. Anat.* **132:** 431–450.

KELLER, R. 1977. Comparative electrophoretic studies of crustacean neurosecretory hyperglycemic and melanophore stimulating hormones from isolated sinus glands. *J. Comp. Physiol.* **122:** 359–373.

KLEINHOLZ, L. H. 1974. A method for removal of the sinus gland from the eyestalk of crustaceans. *Biol. Bull.* **93:** 52–55.

LAKE, P. S. 1970. Histological and histochemical observations of the cephalic neurosecretory system of the crab *Paragrapsus gaimardii* (Milne-Edwards). *Proc. R. Soc. Tasmania* **105:** 87–96.

LAKE, P. S., AND J. E. ONG. 1972. Observations on the organ of Bellonci of the shrimp *Paratya tasmaniensis* Riek (Crustacea Decapoda Atyidae) with particular reference to the structure of the onion body cells. *Aust. J. Zool.* **20:** 215–234.

MARTIN, G. 1972. Contribution à l'étude ultrastructurale de la glande du sinus de l'Oniscoïde *Porcellio dilatatus* Brandt. *C. R. Hebd. Séances Acad. Sci. Sér. D Sci. Nat., Paris* **275:** 839–842.

MATSUMOTO, K. 1958. Morphological studies on the neurosecretion in crabs. *Biol. J. Okayama Univ.* **4:** 103–176.

MIYAWAKI, M. 1956. Histological observations on the incretory elements in the eyestalk of a Brachyura, *Telmessus cheiragonus. J. Fac. Sci. Hokkaido Univ. Ser. VI Zool.* **12:** 325–332.

NAKAMURA, K. 1974. Studies on the neurosecretion of the prawn *Penaeus japonicus* Bates. I. Positional relationships of the cell groups located on the supraoesophageal and the optic ganglions. *Mem. Fac. Fish. Kagoshima Univ.* **23:** 173–207.

PANOUSE, J. B. 1944. L'action de la glande du sinus sur l'ovaire chez la crevette *Leander. C. R. Hebd. Séances Acad. Sci. Paris* **218:** 293–294.

PANOUSE, J. B. 1946. Recherches sur les phénomènes humoraux chez les crustacés. L'adaptation chromatique et la croissance ovarienne chez la crevette *Leander serratus. Ann. Inst. Oceanog.* **32:** 65–147.

PASSANO, L. M. 1951a. The X-organ-sinus gland neurosecretory system in crabs. *Anat. Rec.* **111:** 502.

PASSANO, L. M. 1951b. The X-organ, a neurosecretory gland controlling molting in crabs. *Anat. Rec.* **111:** 559.

PASSANO, L. M. 1953. Neurosecretory control of molting in crabs by the X-organ-sinus gland complex. *Physiol. Comp. Oecol.* **3:** 155–189.

PASTEUR, C. 1958. Influence de l'ablation de l'organe X sur le comportement chromatique de *Leander serratus* (Pennant). *C. R. Hebd. Séances Acad. Sci. Paris* **246:** 320–322.

PYLE, R. W. 1943. The histogenesis and cyclic phenomena of the sinus gland and X organ in Crustacea. *Biol. Bull.* **85:** 87–102.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17:** 19–58.

SCUDAMORE, H. H. 1942. Influence of the sinus gland upon certain metabolic changes associated with molting in the crayfish *Cambarus immunis*. *Anat. Rec.* **84:** 515.

SHIVERS, R. R. 1967. Fine structure of crayfish optic ganglia. *Univ. Kans. Sci. Bull.* **10:** 677–733.

SMITH, G., AND E. NAYLOR. 1972. The neurosecretory system of the eyestalk of *Carcinus maenas* (Crustacea Decapoda). *J. Zool. Lond.* **166:** 313–321.

STEPHENS, G. C. 1951. A molt inhibiting factor in the central nervous system of the crayfish *Cambarus* sp. *Anat. Rec.* **111:** 572–573.

STROLENBERG, G. E. C. M. 1979. Functional aspects of the sinus gland in the neurosecretory system of the crayfish *Astacus leptodactylus*: an ultrastructural approach. *Thesis Cath. Univ. Nijmegen.* Offset Thoben, Nijmegen, The Netherlands. 140 pp.

STROLENBERG, G. E. C. M., C. BELLON-HUMBERT, F. VAN HERP, AND A. VAN WORMHOUDT. 1977. Etude ultrastructurale des granules de neurosécrétion dans la glande du sinus de *Palaemon serratus. C. R. Hebd. Séances Acad. Sci. Sér. D Sci. Nat. Paris* **284:** 1123–1126.

STROLENBERG, G. E. C. M., F. VAN HERP, A. VAN WORMHOUDT, AND C. BELLON-HUMBERT, 1978. Rhythmicity in the neurosecretory activity of the sinus gland of the crayfish *Astacus leptodactylus*. P. 150 in P. H. Gaillard and H. H. Boer, Eds., *Proc. VIIIth Intern. Symp. Comp. Endocrinol.* Elsevier, North-Holland, Amsterdam.

VAN HERP, F. 1972. Fysiologische, biochemische en histochemische aspecten van de hydrolasen bij de rivierkreeft *Astacus leptodactylus. Thesis Cath. Univ. Nijmegen.* Offset Thoben, Nijmegen, The Netherlands. 159 pp.

VAN HERP, F., C. BELLON-HUMBERT, J. T. M. LUUB, AND A. VAN WORMHOUDT. 1977. A histophysiological study of the eyestalk of *Palaemon serratus* (Pennant) with special reference to the impact of light and darkness. *Arch. Biol.* **88:** 257–278.

VAN HERP, F., C. BELLON-HUMBERT, AND A. J. M. DE GROOT. 1979. Presence and development of a sensory formation in the eyestalk of the crayfish, *Astacus leptodactylus* (Nordmann, 1842) during its embryonic, larval and adult life. *J. Morphol.* **161:** 39–51.

VAN HERP, F., AND H. J. M. VAN BUGGENUM. 1979. Immunocytochemical localization of hyperglycemic hormone (HGH) in the neurosecretory system of the crayfish *Astacus leptodactylus. Experientia* **35:** 1527–1528.

VAN WORMHOUDT, A., F. VAN HERP, C. BELLON-HUMBERT, G. E. C. M. STROLENBERG, AND D. VENEMA. 1978. Cyclic variations of the glucose concentration in the hemolymph and studies on the hyperglycemic hormone HGH of *Palaemon serratus* (Crustacea Natantia). P. 174 in P. H. Gaillard and H. H. Boer, Eds., *Proc. VIIIth Intern. Symp. Comp. Endocrinol.* Elsevier North-Holland, Amsterdam.

WATSON, M. L. 1968. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.* **4:** 475–478.

Reference: *Biol. Bull.*, **160:** 31–42. (February, 1981)

VARIABILITY IN BROODING ACTIVITY IN THE STALKED BARNACLE *POLLICIPES POLYMERUS*

ROBERT L. CIMBERG[1]

Department of Biological Sciences, University of Southern California,
Los Angeles, California, U. S. A. 90007

Abstract

Reproductive activity in the barnacle, *Pollicipes polymerus*, was studied at sites along the west coast of North America and in the laboratory. Patterns of seasonal brooding activity reveal two physiological races: a northern one with maximum brooding activity at cold seawater temperatures (14°C or less) and a southern one which broods most at warmer temperatures (20°C). The distribution of these two races corresponds, respectively, to the cold and warm temperate zones located north and south of Point Conception.

Laboratory experiments support field results that the northern physiological race broods at lower temperatures than the southern physiological race and that seasonal brooding activity is controlled more by water temperature than by food. A specific temperature level does not account for the onset of seasonal brooding activity as well as does a change in temperature toward the optimum level for each physiological race. A possible reason that food does not regulate seasonal reproductive activity in *P. polymerus* is that this large species has sufficient food reserves as compared with smaller barnacles found at higher intertidal levels.

Introduction

Pollicipes (Mitella) polymerus, Sowerby, 1833, is the common intertidal stalked barnacle along the Pacific coast of North America from Susk, British Columbia (Pilsbry, 1907), to Punta Santa Dominga, Baja California Sur (personal observations). Reproductive activity in this and other intertidal invertebrates varies with space and time and has been attributed to environmental and genetic differences. Previous researchers have studied seasonal reproductive activity in *P. polymerus* by determining the percentage of adults brooding. They noted that periods of high brooding activity correspond with changes in water temperature. Brooding at sites in the cold temperate zone north of Point Conception is greatest during the summer (Hilgard, 1960; Cimberg, 1973; Hand *et al.*, 1973; Lewis, 1975a), whereas in the warm temperate waters south of Point Conception brooding increases during the winter (Straughan, 1971; Cimberg, 1973). This seasonal shift suggests that brooding occurs at an optimal temperature (Cimberg, 1973; Hand *et al.*, 1973), perhaps around 14°C (Cimberg, 1973), reached during summer in the cold temperate and during winter in the warm temperate.

An alternative hypothesis is that some other variable (such as food), which correlates with water temperature, could be the proximal environmental stimulus that triggers the onset of seasonal reproduction. Plankton abundance regulates

Received 1 December 1979, accepted 5 November 1980.

---

[1] Present Address: VTN Oregon, Inc., Environmental Sciences, 8285 S. W. Nimbus Ave., Suite 151, Beaverton, Oregon 97005.

seasonal reproductive activity in several barnacle species (Hines, 1978) and increases during the spring as *P. polymerus* broods at Friday Harbor (Lewis, 1975a).

Reproductive activity varies on a small spatial scale with changes in tidal level in barnacles (Crisp, 1950, 1959) and other invertebrates. This might occur in *P. polymerus*. On a larger spatial scale, variability in reproductive activity within a species whose populations are geographically separated and exposed to different environmental conditions has been attributed to genetic differences (Sastry, 1975). Such variability might occur between populations of *P. polymerus* from the warm and cold temperate zones.

This study further investigated patterns of and factors regulating reproductive activity in the barnacle *P. polymerus*. Answers to the following questions were sought: (1) Does seasonal reproductive activity at sites in both the cold and warm temperature zones fit the model of an optimal temperature for brooding activity? (2) Does water temperature, more than food, control seasonal reproductive activity? (3) What is the relative importance of tidal exposure on reproductive activity? (4) Is there reproductive evidence for genetic differences between populations?

MATERIALS AND METHODS

Field studies

Seasonal brooding activity was studied at a cold temperate site near the Pismo Beach pier (35.10° N, 120.37° W) and at warm temperate sites at Bird Rock, Santa Catalina Island, (33.21° N, 118.20° W) and at Latigo Point (34.02° N, 118.38° W) (Fig. 1) during 1972–1977. *P. polymerus* was sampled throughout the

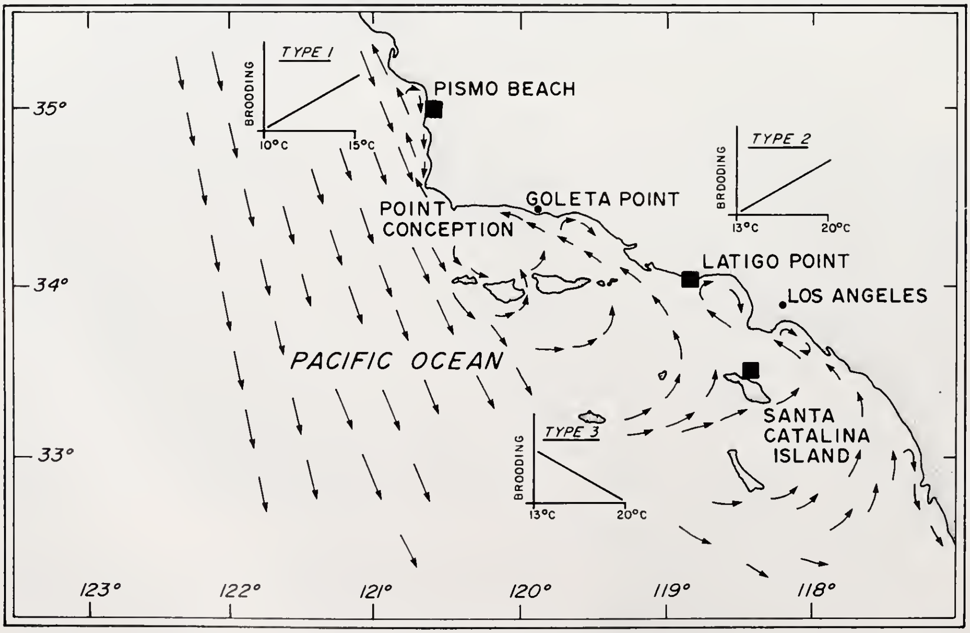

TYPE 1
BROODING
10°C
15°C
PISMO BEACH
35°
TYPE 2
BROODING
13°C
20°C
GOLETA POINT
POINT CONCEPTION
LATIGO POINT
34°
LOS ANGELES
PACIFIC OCEAN
SANTA CATALINA ISLAND
TYPE 3
BROODING
13°C
20°C
33°
123°
122°
121°
120°
119°
118°

FIGURE 1. Map of the region around Point Conception, California, showing study sites (solid squares) and nearshore current patterns during the oceanic and upwelling seasons (after Pirie *et al.*, 1975).

year. Each sample consisted of a cluster of 10–20 adults with a capitulum height (base of lower latera to tip of tergum) greater than 12 mm, the minimum size at which brooding usually occurs (Barnes and Reese, 1960; Straughan, 1971; Cimberg, 1973). Only animals from clusters were sampled, to insure that cross-fertilization could have occurred. The effect of tidal exposure on brooding was investigated by taking samples from different intertidal levels. The *Pollicipes* zone (that portion of the intertidal region inhabited by adult *Pollicipes*) was divided into two equal vertical sections; 1–4 samples were taken from both the upper and lower levels, thereby totaling 20–160 animals sampled per month at each site. In the laboratory, animals were dissected and the percentage of adults brooding was determined for each sample.

Water temperature data for the respective sites were collected from nearby shore stations: for Pismo Beach, from daily readings at Port San Luis (Physical and Chemical Oceanographic Data Facility, Scripps Institute of Oceanography, unpublished data); for Santa Catalina Island, from daily readings at the Catalina Marine Science Center pier for 1972–1974 (Catalina Marine Science Center, unpublished data) and from weekly means taken from the kelp bed canopy at Bird Rock for 1976 (James Coyer, University of Southern California, personal communication); and for Latigo Point, from daily measurements at Zuma Beach County Park (Los Angeles County Department of Parks and Recreation, unpublished data).

As an alternative to measuring plankton abundance, body weights of barnacles have been used to estimate food reserves (Barnes *et al.*, 1963; Hines, 1978). In this study the soft tissues of the capitulum and peduncle were dissected from their respective external shells and cuticular sheaths and dried at 60°C to constant weight. These weights were standardized for size by dividing by the cube of the rostral-carinal length (Lewis, 1975a), which eliminated the effect of size (Cimberg, unpublished data). Body weights were determined for Santa Catalina Island and Latigo Point animals sampled in 1976 during March, June, October, and December. Using a stratified random method to sample the entire tidal range which *P. polymerus* inhabits, a total of 335 animals were collected from the two sites.

Laboratory experiments

Laboratory experiments were conducted in nine 21-l aquaria in which temperature and food could be varied independently. Each tank was equipped with a bubble curtain to provide vigorous and uniform circulation and a heater to maintain the temperature at the desired experimental level (±1°C). Food abundance was regulated daily in each tank by adding a specified amount of freshly hatched brine shrimp nauplii (*Artemia salina*). All experiments were run under a 12 h light/12 h dark cycle. Seawater was collected on the Palos Verdes Peninsula of Los Angeles, filtered through 0.22 μm Millipore filters, and stored at 6°C in glass containers. To control bacterial growth, seawater was changed every 5 days and Maracyn antibiotics (5 mg minocycline and 100 mg erythromycin) were added daily.

Experimental animals were collected from the middle of the *Pollicipes* zone by chipping off pieces of rock or mussel to which the barnacle clusters were attached. These animals were placed in each tank so that their feeding apparatuses faced into the water current. The animals were maintained for 24 h at the temperature at which they were collected. During the subsequent 7 days, the temperature of each tank was gradually changed to the prescribed experimental level. Each experiment ran for 30 days, after which the percentage of adults brooding was determined. Because development of the brooding embryos takes 20–31 days (Lewis, 1975b), nearly all embryos present at the end of the experimental period would have been deposited in the mantle (brood) cavity under laboratory conditions.

Two experiments were conducted. Experiment 1 was designed to measure the relative importance of temperature and food on brooding. Three temperatures (10, 15, and 20°C) were tested in combination with three different daily rations of food in the ratio of 0:1:4. This experiment was conducted from 12 October through 10 November 1976, using Pismo Beach animals. Experiment 2 tested for genetic differences, by comparing brooding responses to the same temperatures in animals from two different sites. Each tank received the same amount of *Artemia*, but was set at a different temperature: 8, 10, 12, 14, 16, 18, 20, or 22°C. This experiment was conducted from 18 July to 16 August 1977, using animals from Santa Catalina Island and La Jolla. La Jolla animals (32.50° N, 117.17° W) were used in place of Latigo Point barnacles since not enough experimental animals could be collected from the latter site.

RESULTS

Field studies

Brooding at both tidal levels was compared with water temperature and season (Fig. 2). Although brooding cycles at each site corresponded both with water temperatures (based on a Spearman rank correlation coefficient; Nie *et al.*, 1975) and season, these results differed among sites. At Pismo Beach, brooding occurred during the summer and was highest at 15°C, since brooding activity increased significantly at water temperatures between 12 and 15°C ($r_s = +0.5070$, N = 104, $P < 0.001$), but decreased significantly at temperatures above 15°C $r_s = -0.6187$, N = 10, $P < 0.05$). At Latigo Point, which is exposed to warmer temperatures than Pismo Beach, barnacle brooding was highest during the summer and increased significantly as temperatures increased and approached 20°C ($r_s = +0.4666$, N = 136, $P < 0.001$). Finally, at Santa Catalina Island, where water temperatures are comparable to those at Latigo Point, brooding was highest during the winter, increasing significantly as temperatures decreased and approached 13°C ($r_s = -0.4897$, N = 183, $P < 0.001$). Comparisons of yearly reproductive cycles at Santa Catalina Island showed that a sharp increase in brooding activity each fall corresponded more closely to a change in water temperature than to any particular temperature level (Fig. 2). This fall increase occurred when temperature declined from 18 to 16°C in 1973 as compared to 21 to 19°C in 1976.

Maximum brooding in the higher and lower portions of the *Pollicipes* zone occurred during the same season at each respective site. However, other aspects of the reproductive cycle differed with tidal elevation (Fig. 2). Brooding was significantly greater in the lower than higher portion of the *Pollicipes* zone at Pismo Beach (+15%, N = 14, $P < 0.05$), Latigo Point (+14%, N = 17, $P < 0.05$), and Santa Catalina Island (+17%, $n = 31$, $p < 0.05$), based on a t test for paired comparisons (Nie *et al.*, 1975). In addition, populations brooded earlier in the year and for a longer duration at lower tidal elevations at each site.

Body weights, analyzed using Duncan's multiple range test (Nie *et al.*, 1975), varied significantly ($P < 0.05$) during the year at each site (Fig. 3). Values at Latigo Point were significantly greater ($P < 0.05$) in October than in March, June, or December, whereas at Santa Catalina Island body weights were significantly greater ($P < 0.05$) in June than during the other 3 months.

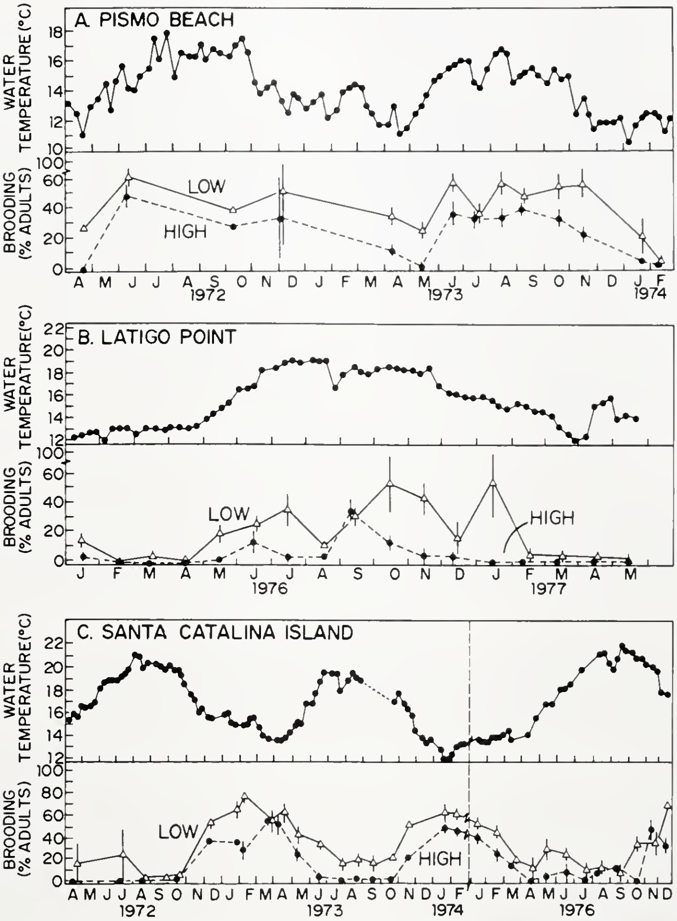


FIGURE 2. Comparison of seasonal brooding activity with average weekly seawater temperatures for populations of *Pollicipes polymerus* at Pismo Beach (A), Latigo Point (B), and Santa Catalina Island (C). Vertical lines represent standard error of the means for high (circles) and low (triangles) intertidal samples.

Changes in brooding activity at each site were compared with water temperature and body weights to determine the relative importance of these two factors on brooding in the field (Fig. 3). Brooding at Latigo Point was greatest when both temperature and food reserves were highest. Brooding at Santa Catalina Island occurred as food reserves were low, but as temperatures approached the optimum level. Correlation coefficients (Spearman rank) were calculated between brooding activity and each of these two independent variables, using data from both sites combined. Brooding was correlated more strongly with a change in temperature, toward the apparent optimal level ($r_s = +0.29$), than with body weight ($r_s = +0.22$).

Laboratory experiments

Results of experiment 1 (Fig. 4) indicated that water temperature had a greater effect on brooding than did food abundance. Food affected brooding under some

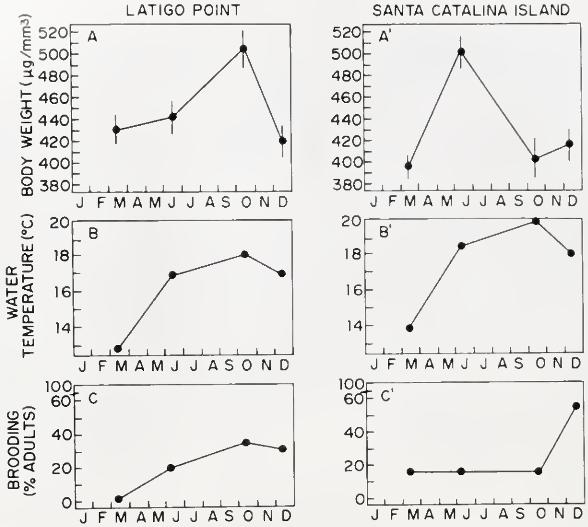
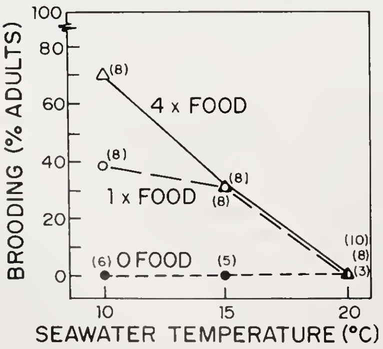


FIGURE 3. Comparisons of *Pollicipes polymerus* brooding activity with seawater temperature and body weight (means and standard errors) at Latigo Point (A, B, C) and Santa Catalina Island (A′, B′, C′).

conditions: unfed animals did not brood, and at the temperature of maximum brooding activity (10°C) food had a significant effect ($\chi^2 = 8.04$, DF = 2, $P < 0.05$). But for all temperatures combined, the effect of food was not significant ($\chi^2 = 5.33$, DF = 2, $P < 0.05$). On the other hand, water temperature had a significant effect on brooding ($\chi^2 = 11.14$, DF = 2, $P < 0.005$) for all food levels combined. Results of experiment 2 (Fig. 5) indicate that barnacles from La Jolla and Santa Catalina Island responded significantly differently ($P < 0.05$) to the same water temperatures, based on a Kolmogorov-Smirnov test (Sokal and Rohlf, 1969); Santa Catalina Island barnacles brooded at significantly lower temperatures than animals from La Jolla.



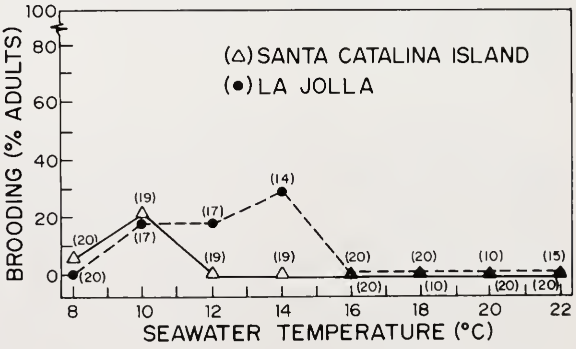


FIGURE 4. (Left) Results of experiment 1, testing the effects of water temperatures (10, 15, and 20°C) and food abundance (in the ratio 0:1:4) on brooding activity of *Pollicipes polymerus* from Pismo Beach, California. Numbers in parenthesis represent sample size per treatment.

FIGURE 5. (Right) Results of experiment 2, comparing brooding responses of La Jolla and Santa Catalina Island populations of *Pollicipes polymerus* to the same water temperatures. Numbers in parenthesis represent sample size per treatment.

Discussion

Brooding and water temperature

Brooding at Pismo Beach and Santa Catalina Island occurs as temperatures approach approximately 14°C, supporting the optimum temperature model proposed independently by Cimberg (1973) and Hand *et al.* (1973). Latigo Point, where brooding increases as temperatures approach 20°C, is the first reported site in which brooding of this species does not increase as temperatures approach ~14°C. Examination of data from all *P. polymerus* studies revealed three types of brooders (Fig. 6). Type 1 animals, located at all sites north of Point Conception, brood most during the summer as temperatures approach ~14°C. Type 2 barnacles at Latigo Point, south of Point Conception, brood most during the summer as temperatures increase and approach 20°C. Type 3 animals are found at Santa Catalina Island and Goleta Point, also south of Point Conception, but brood most during the winter as temperatures decrease and approach 13°C.

Type 1 and 3 brooders are similar in the temperature (~14°C), though not season, at which brooding occurs. Type 3 animals could have been derived from type 1 populations (Fig. 1). Both Santa Catalina Island and Goleta Point are exposed to portions of the south flowing California Current during the oceanic (March through August) and upwelling seasons (July through November) (Pirie *et al.*, 1974). During these periods *P. polymerus* larvae are released into the water north of Point Conception. Since the current has an average velocity of 0.25 m/s (Jennings and Schwartlose, 1960), the larvae could be transported 1014 km during their 42 day planktonic period (Lewis, 1975b). As the direct distances from Pismo Beach to Goleta Point and Santa Catalina Island are approximately 145 and 385 km respectively, larvae could reach these sites even if the route was indirect and/or the transport slower.

An alternative relationship among brooding types is that the Latigo Point population (type 2) is a southern extension of the cline composed of type 1 populations (Fig. 6). Maximum brooding in this cline occurs at progressively higher temperatures with correspondingly lower latitudes. This theory does not explain why animals at Goleta Point and Santa Catalina Island (type 3), exposed to essentially

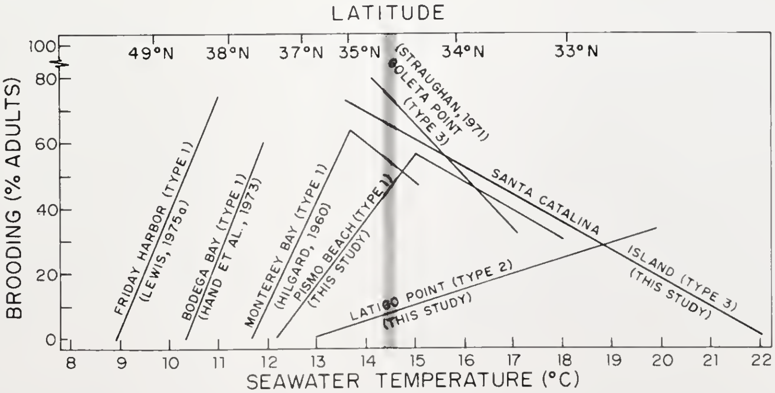


FIGURE 6. Comparison of brooding activity with seawater temperature for *Pollicipes polymerus* populations at sites along the west coast of North America north and south of Point Conception (shaded line).

the same temperatures as Latigo Point animals, have a different pattern and brood during the winter. Nor does it explain why brooding decreases at temperatures higher than ~14°C at Pismo Beach and Monterey Bay.

Two kinds of brooders therefore can be identified from this analysis. One, consisting of type 1 and 3 animals, broods at relatively cold temperatures (~14°C or less), regardless of season (Fig. 1); the other, type 2 animals, broods at relatively warm temperatures (20°C). The presence of both kinds of brooders in southern California, exposed to essentially the same water temperatures and yet brooding at different times of the year, indicates genetic rather than environmental differences. Therefore, the cold-water brooders (types 1 and 3) are considered a northern physiological race and the warm-water brooders (type 2) a southern physiological race. Laboratory results indicated that Santa Catalina Island animals (the northern physiological race) brood at significantly lower temperatures than La Jolla barnacles (considered to be of the southern physiological race), even though they are exposed to essentially the same temperatures in the field. This experiment therefore supports field studies regarding the presence of two physiological races.

Physiological races, determined from reproductive studies, have been identified in the American oyster *Crassostrea virginica* (Loosanoff and Nomejko, 1951; Loosanoff, 1969) and the barnacle *Balanus balanoides* (Barnes and Barnes, 1976). Physiological races of these three species, which all have large distributional ranges, occur in different biogeographic provinces. Such races can be difficult to detect, since in *P. polymerus* and *C. virginica*, different races breed during the same season but at different temperatures due to genetic differences; and in *P. polymerus*, the same physiological race will breed at the same temperatures but in different seasons due to environmental differences.

Seasonal brooding and other factors

Brooding at Latigo Point is greatest in October, when body weights are maximal and temperatures apparently optimal. Brooding at Friday Harbor begins in the spring as temperatures and phytoplankton abundance both increase (Lewis, 1975a). Because both factors change at the same time at these sites, their relative importance is difficult to determine. Santa Catalina Island represents a good natural field experiment in that maximum brooding occurs in the winter when food reserves are minimal but temperatures apparently favorable. Correlation coefficients from field data indicate that seasonal brooding corresponds to changes in water temperature better than to changes in food reserves. Laboratory experiment 1 supports field results that temperature has a greater effect than food on seasonal brooding.

The relative importance of water temperature and food on the reproduction of various eastern Pacific, warm temperate, littoral barnacles has an interesting relationship with tidal elevation. Brooding activity in the highest intertidal barnacle (*Chthamalus fissus*) is regulated by food abundance; temperature has no significant effect (Hines, 1978). This species, the smallest of the four common intertidal barnacles along this coast, begins to die after 2 weeks without food in the laboratory (Diane Perry, University of Southern California, personal communication), indicating low food reserves. Seasonal reproduction of the lower barnacle (*Balanus glandula*), is controlled by low seasonal temperatures (Hines, 1978). This larger species lives longer than *C. fissus* when starved (Diane Perry, personal communication), which suggests the presence of greater food reserves. These reserves are stored before and then depleted during the brooding season (Hines, 1978), indicating that food abundance might affect brooding. Seasonal reproduction in the two lowest intertidal barnacles is regulated by water temperature; both *P. polymerus* (this study) and *Tetraclita squamosa* (Hines, 1978) breed at an optimal temperature, and are apparently not affected by food abundance. These two species are larger than the others and have greater nutrient reserves; *P. polymerus* can live for at least 4 weeks without food (this study), and nutrient storage in *T. squamosa* does not decrease during the reproductive season (Hines, 1978). Thus, the larger barnacle species along the coast inhabit lower tidal elevations and apparently store greater food reserves. Greater nutrient storage may explain why food is less important than water temperature in regulating seasonal brooding in these species than in smaller barnacles at higher tidal levels.

Light influences seasonal reproduction in the barnacle *Balanus balanoides*, (Barnes, 1963) and inhibits *P. polymerus* feeding in the laboratory (personal observations). However, light does not appear to be the primary factor controlling *P. polymerus* seasonal reproduction, since populations at Latigo Point and Santa Catalina Island, essentially at the same latitude and exposed to the same light regimens, brood 6 months apart.

Temperature control of reproduction

The factor triggering the onset of seasonal brooding might not be a specific water temperature but a change in temperature. Brooding at Santa Catalina Island increased sharply each fall at different temperature levels, but always with a temperature decline. Brooding at sites north of Point Conception occurred at different temperatures, but always as temperatures changed towards an optimum. Maximum brooding at Santa Catalina Island and Pismo Beach did not occur at the same temperatures in the laboratory as in the field for the respective sites, but did occur with a change in temperature. Brooding in *P. polymerus* therefore could be regulated by a change in temperature, as has been found in the reproductive processes of other marine invertebrates (Giese and Pearse, 1974). The possibility that a temperature change might control brooding activity does not preclude the presence of physiological races reproducing at different temperatures. It does indicate that such a triggering mechanism operates within the individual activity ranges of each race. A change of temperature towards an optimum is therefore hypothesized to trigger the onset of seasonal brooding in each race.

The season of maximum reproductive activity can change with a shift in the time of year when the optimal temperature for brooding occurs. Santa Catalina Island and Goleta Point populations of *P. polymerus* breed 6 months apart from populations north of Point Conception, but as temperatures approach the same level (Fig. 1). The same shift is noted between populations of the northern anchovy *Engraulis mordax* found north (Richardson, 1973) and south (Brewer, 1978) of Point Conception. On a smaller geographic scale, the barnacle *Tetraclita squamosa* normally broods during the summer in Morro Bay as temperatures approach 15°C (Hines, 1978), but 2 miles away near a thermal outfall (13°C above ambient) these barnacles brood in the winter when decreasing temperatures approach 15°C. Finally, *P. polymerus* populations located further south at Punta Santa Dominga, Baja California Sur, brood at approximately 20°C during the winter (Cimberg, unpublished data), 6 months out of phase with, but at the same temperatures as, populations of the southern race at Latigo Point.

Brooding activity and tidal elevation

Maximum brooding activity of *P. polymerus* occurs during the same season for both high and low intertidal populations at each site, but the percentage of adults brooding was significantly greater per month at lower than higher tidal elevations. Although Lewis (1975a) reported no significant differences in the percentage of adults brooding at different tidal elevations at Friday Harbor, a recalculation of her data using a *t* test for paired comparisons (Nie *et al.*, 1975), during the months in which brooding did occur, shows that brooding activity is significantly greater (+9% difference, $P < 0.05$) at lower tidal levels. Animals at lower tidal elevations also brood earlier and during more months of the year at each site in the present study. Populations at Santa Catalina Island and Pismo Beach brood throughout the year at lower elevations, but only during part of the year at higher intertidal levels.

The relationship between the level of the intertidal in which brooding first occurs in *Pollicipes* and other invertebrates, and the factor that regulates seasonal reproductive activity, suggests that changes in reproductive activity with both space and time could be due to the duration and intensity of a single factor. The barnacle *Chthamalus stellatus* (Crisp, 1950), the clam *Hiatella* (Hunter, 1949), and the limpet *Acmaea scabra* (Sutherland, 1970) all breed earlier at lower than higher tidal levels. Seasonal reproductive activity in *C. stellatus* (Patel and Crisp, 1960), *Hiatella, A. scabra*, and *P. polymerus* (this study), is controlled by temperature and/or food abundance. In contrast, seasonal reproductive activity in the barnacle *Balanus balanoides* occurs earlier at high tidal elevations (Crisp, 1959) and is influenced by light (Barnes, 1963) and possibly air temperature (Barnes and Barnes, 1976). Therefore, the onset of reproductive activity in these species occurs initially in that level of the intertidal zone having the longest exposure (duration) to the factor(s) that controls seasonal reproductive activity. In turn, seasonal reproductive activity in these species occurs with changes in the intensity (dosage) of the controlling factor(s). Therefore, patterns of reproductive activity at a single locality occur with space (tidal height) and time (season) and could be regulated, respectively, by the duration and intensity of the controlling factor.

Biogeography of races

For many taxa, Point Conception is a biogeographical boundary between the cold and warm temperate zones (Brusca and Wallerstein, 1979). Newman (1979) noted that of all 26 species of near-shore temperate barnacles, *P. polymerus* is the only species whose northern or southern limit does not end near Point Conception; however, this study indicates that the distribution of the two physiological races of *P. polymerus* is interrupted there. Electrophoretic studies indicate that this area also acts as a boundary between genetically dissimilar populations of the isopod *Ligia occidentalis* (McGill, 1978) and the sculpin *Clinocottus analis* (Swank, 1979).

The distribution of the *P. polymerus* races is associated with the warm and cold temperate waters; however, offshore populations on Santa Catalina Island are exposed to essentially the same water temperatures as Latigo Point animals on the mainland, but are genetically more similar (based on reproductive studies) to populations in colder waters north of Point Conception. Similar results based on electrophoretic patterns were reported for populations of *C. analis* (Swank, 1979) and *L. occidentalis* (McGill, 1978). These offshore islands are exposed to currents originating from cold waters north of Point Conception (Fig. 1) but have temperatures similar to those along the southern California mainland. Thus, at certain sites in the transition zone between the two regions, the distribution of these races corresponds better to current patterns, which affect larval dispersal, than to temperatures, which affect the adult's physiology.

The relationship between temperatures within a species' biogeographic range and temperatures for reproduction has been noted by Hutchins (1947). He hypothesized that the minimum and maximum temperatures for reproduction among northern hemisphere shallow water species corresponded, respectively, with the summer temperatures at the northern boundary and the winter temperatures at the southern boundary. Molluscan data (Golikov and Scarlato, 1973) supports this model; the Pacific-Asiatic-low-boreal (cold temperate) species reproduce between 6 and 14°C, whereas the Pacific-Asiatic-subtropical-low-boreal (warm temperate) species reproduce between 14 and 20°C. Field data indicate that *P. polymerus* populations of the cold temperate race brood most actively between 11 and 14°C, and the warm temperate race broods at 20°C. Field data near the distributional boundaries are needed to determine more accurately the entire temperature range of brooding activity for populations of both races.

Acknowledgments

This study was part of a dissertation submitted to the Department of Biological Sciences at the University of Southern California. Portions of this study were supported by the Society of Sigma Xi (Grants-in-aid of Research), University of Southern California (Biomedical Support Grant), and a contract from the American Petroleum Institute (No. 903022) to Dr. Dale Straughan. I gratefully acknowledge: S. Allerton, M. Halula, J. McConaugha, G. Morey-Gaines, and D. Rainwater for laboratory support; B. Hart, S. Kottmier, and T. Smith for field assistance; R. Smith for expertise on statistics; P. Fishman, J. Kastendiek, W. Newman, E. Norse, and two anonymous reviewers for their suggestions on the manuscript; Mardel Laboratories for antibiotics; Marineland of the Pacific for seawater; and S. Donin and J. Duncan for typing the manuscript. I am indebted to B. Abbott, G. Bakus, K. Fauchald, D. Perry, A. Siegman, and the late R. Stone for their guidance and especially to R. Brusca and R. Zimmer for their support.

LITERATURE CITED

Barnes, H. 1963. Light, temperature and the breeding of *Balanus balanoides*. *J. Mar. Biol. Assoc. U. K.* **43:** 117–127.

Barnes, H., and M. Barnes. 1976. The rate of development of the embryos of *Balanus balanoides* (L.) from a number of European and American populations and the designation of local races. *J. Exp. Mar. Biol. Ecol.* **24:** 251–269.

Barnes, H., M. Barnes, and D. M. Finlayson. 1963. The seasonal changes in body weight, biochemical composition, and oxygen uptake of two common boreo-arctic cirripeds, *Balanus balanoides* and *Balanus balanus*. *J. Mar. Biol. Assoc. U. K.* **43:** 185–211.

Barnes, H., and E. S. Reese. 1960. The behaviour of the stalked intertidal barnacle *Pollicipes polymerus* J. B. Sowerby, with special reference to its ecology and distribution. *J. Anim. Ecol.* **29:** 169–185.

Brewer, G. D. 1978. Reproduction and spawning of the northern anchovy *Engraulis mordax*, in San Pedro Bay, California. *Calif. Fish Game Bull.* **64(3):** 175–184.

Brusca, R. C., and B. R. Wallerstein. 1979. Zoogeographic patterns of idoteid isopods in the northeast Pacific, with a review of shallow water zoogeography of the area. *Biol. Soc. Wash. Bull.* **3:** 67–105.

Cimberg, R. L. 1973. The effects of exposure to chronic natural oil pollution on the reproductive biology of *Pollicipes polymerus*. Pp. 179–202 in D. Straughan, Ed., *Sublethal effects of natural chronic exposure to petroleum in the marine environment*. First year report to the American Petroleum Institute, Washington, D. C.

Crisp, D. J. 1950. Breeding and distribution of *Chthamalus stellatus*. *Nature* **166:** 311–312.

CRISP, D. J. 1959. Factors influencing the time of breeding of *Balanus balanoides*. *Oikos* **10:** 275–289.

GIESE, A. C., AND J. S. PEARSE. 1974. Introduction: General principles. Pp. 1–49 in A. C. Giese and J. S. Pearse, Eds., *Reproduction of marine invertebrates*. Vol. 1., Academic Press, New York.

GOLIKOV, A. N., AND O. A. SCARLATO. 1973. Methods for indirectly defining optimum temperatures of inhabitance for marine cold blooded animals. *Mar. Biol.* **20:** 1–5.

HAND, C., J. D. STANDING, AND J. C. RUTHERFORD. 1973. San Francisco oil spill, effects on reproduction in intertidal invertebrates (Project No. 15080). US Environmental Protection Agency, Washington, D. C. 46 pp.

HILGARD, G. H. 1960. A study of reproduction in the intertidal barnacle *Mitella polymerus*, in Monterey Bay, California. *Biol. Bull.* **119:** 169–188.

HINES, A. H. 1978. Reproduction in three species of intertidal barnacles from central California. *Biol. Bull.* **154:** 262–281.

HUNTER, W. R. 1949. The structure and behavior of *Hiatella gallicana* (Lamarck) and *H. artica* (L) with special reference to the boring habit. *Proc. R. Soc. Edinb. Sect. B. Biol. Sci.* **63(3):** 271–289.

HUTCHINS, L. W. 1947. The bases for temperature zonation in geographical distribution. *Ecol. Monogr.* **17:** 325–335.

JENNINGS, F. D., AND R. A. SCHWARTLOSE. 1960. Measurements of the California Current in March 1958. *Deep Sea Res.* **7:** 42–47.

LEWIS, C. A. 1975a. *Reproductive biology and development of the gooseneck barnacle*, Pollicipes polymerus, *with special emphasis on peristaltic constrictions in the fertilized egg*. Ph.D. dissertation, University of Alberta, Edmonton, Canada. 320 pp.

LEWIS, C. A. 1975b. Some observations on factors affecting embryonic and larval growth of *Pollicipes polymerus* (Cirripedia: Lepadomorpha) *in vitro*. *Mar. Biol.* **32:** 127–139.

LOOSANOFF, V. L. 1969. Maturation of gonads of oysters, *Crassostrea virginica*, of different geographic areas subjected to relatively low temperatures. *Veliger* **11:** 153–163.

LOOSANOFF, V. L., AND C. A. NOMEJKO. 1951. Existence of physiologically different races of oyster, *Crassostraea virginica*. *Biol. Bull.* **101:** 151–156.

MCGILL, T. J. 1978. *Genetic divergence of mainland and insular populations of* Ligia occidentalis (*Oniscoidea: Isopoda*). Ph.D. dissertation, University of California, Santa Barbara. 100 pp.

NEWMAN, W. A. 1979. California transition zone: Significance of short-range endemics. Pp. 399–416 in A. Boucot and J. Gray, Eds., *Historical biogeography, plate tectonics, and the changing environment*. The 37th Annual Biological Colloquium, Oregon State Press, Corvallis.

NIE, N. H., C. H. HULL, J. G. JENKINS, K. STEINBRENNER, AND P. BRENT. 1975. *Statistical package for the social sciences*. McGraw Hill, New York. 675 pp.

PATEL, B., AND D. J. CRISP. 1960. The influence of temperature on the breeding and the moulting activities of some warm water species of operculate barnacles. *J. Mar. Biol. Assoc. U. K.* **39:** 667–680.

PILSBRY, H. A. 1907. The barnacles (Cirripedia) contained in the collections of the U. S. National Museum. The Lepadidae. *U. S. Natl. Mus. Bull.* **60:** 1–122.

PIRIE, D. M., M. J. MURPHY, AND J. R. EDMISTEN. 1974. California nearshore processes study. Final Report, U. S. Army Corps Engineers, San Francisco District. 30 pp.

RICHARDSON, S. L. 1973. Abundance and distribution of larval fishes in waters off Oregon, May–October 1969, with special emphasis on the northern anchovy, *Engraulis mordax*. *U. S. Natl. Mar. Fish. Serv. Fish. Bull.* **71(3):** 697–711.

SASTRY, A. N. 1975. Physiology and ecology of reproduction in marine invertebrates. Pp. 279–300 in F. J. Vernberg, Ed., *Physiological ecology of estuarine organisms*. University of South Carolina Press, Charleston.

SOKAL, R. R., AND F. J. ROHLF. 1969. *Biometry*. W. H. Freeman and Co., San Francisco. 776 pp.

STRAUGHAN, D. 1971. Breeding and larval settlement of certain intertidal invertebrates in the Santa Barbara Channel following pollution by oil. Pp. 223–244 in D. Straughan, Ed., *Biological and oceanographic survey of the Santa Barbara Channel oil spill 1969–1970. Vol. I. Biology and Bacteriology*. Allan Hancock Foundation, University of Southern California, Los Angeles.

SUTHERLAND, J. P. 1970. Dynamics of high and low populations of the limpet *Acmaea scabra* (Gould). *Ecol. Monogr.* **40:** 169–188.

SWANK, S. E. 1979. Population genetics and evolution of some intertidal fishes of the genus *Clinocottus*. *Ph.D. dissertation, University of Southern California*, Los Angeles, 106 pp.

Reference: *Biol. Bull.*, **160**: 43–54. (February, 1981)

CHLOROPLAST SYMBIOSIS IN A NON-ELYSIID MOLLUSC, *COSTASIELLA LILIANAE* MARCUS (HERMAEIDAE: ASCOGLOSSA (=SACOGLOSSA): EFFECTS OF TEMPERATURE, LIGHT INTENSITY, AND STARVATION ON CARBON FIXATION RATE

KERRY B. CLARK, KATHE R. JENSEN, HUGH M. STIRTS, AND CESAR FERMIN

Department of Biological Sciences, Florida Institute of Technology, Melbourne, FL 32901

ABSTRACT

The hermaeid ascoglossan slug *Costasiella lilianae* possesses functional symbiotic chloroplasts derived from its algal food, *Avrainvillea nigricans.* Symbiotic plastids continued to fix carbon after 65 days starvation, though efficiency of fixation declined about 87%. Chlorophyll level did not decline during this period.

Degeneration of symbiotic plastids involved swelling and delamination of thylakoids, increase in electron density of plastids, and decrease in pyrenoid electron density. Plastids within single cells degenerate at about the same time, suggesting that individual cells phagocytize the entire complement of plastids during a brief period.

Temperature strongly influenced carbon fixation, both in rate of net fixation and in production of alcohol-insoluble photosynthates. The optimum temperature for fixation was 25°C. Photosynthetic rate exhibited saturation at about 500 $\mu\epsilon$ (microeinsteins)$\cdot m^{-2}\cdot s^{-1}$ and substantial fixation occurred at intensities as low as 25 $\mu\epsilon\cdot m^{-2}\cdot s^{-1}$. No inhibition occurred in full sunlight (1500 $\mu\epsilon\cdot m^{-2}\cdot s^{-1}$).

INTRODUCTION

The opisthobranch molluscan order Ascoglossa (=Sacoglossa) appears to be unique among Metazoa in its ability to symbiotically retain functional chloroplasts within cells of the digestive tract (see Trench, 1975, for a recent review). However, there is disagreement on how widespread the symbiosis is within the order. There are three major morphological types of Ascoglossa: the tectibranch (primitively shelled) families (Cylindrobullidae, Volvatellidae, Juliidae, Oxynoidae, and Lobigeridae); the elysioids (Elysiidae), which are shell-less and have parapodia (lateral extensions of the body wall); and the stiligeroids (Caliphyllidae, Hermaeidae, Stiligeridae), which are also shell-less, but lack parapodia and have dorsal papillae (cerata). Of the three types, chloroplast symbiosis has been thought to be limited to the Elysiidae (Muscatine and Greene, 1973; Hinde and Smith, 1974; Trench, 1975; Muscatine *et al.*, 1975; Graves *et al.*, 1979). However, other workers (Taylor, 1967, 1971; Kremer and Schmitz, 1976; Clark and Busacca, 1978) have shown that forms of chloroplast symbiosis do occur in some non-elysiids, though these symbioses are often of short duration. We report here evidence of long-term chloroplast symbiosis in *Costasiella lilianae* (Marcus and Marcus), the second genus and species of Hermaeidae known to retain symbiotic plastids.

Received 6 June 1980; accepted 23 November 1980.

Abbreviations: $\mu\epsilon$, microeinsteins; CPM, counts per minute; DPM, disintegrations per minute; LSC, liquid scintillation counting; AS, alcohol soluble; AI, alcohol insoluble; Chl, chlorophyll.

MATERIALS AND METHODS

Initial stocks of *Costasiella lilianae* were obtained from Bermuda and Key Largo, Florida, from the alga *Avrainvillea nigricans* Decaisne. All experimental stocks were drawn from aquarium cultures maintained with a photoperiod of 18 h light: 6 h dark, irradiance of 50–100 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$, and temperatures of 23–25°C.

Carbon fixation

Photosynthetic rates were estimated by uptake rate of radioisotopic carbon. Each animal was incubated 1 h in 4 ml of membrane-filtered seawater with $NaH^{14}CO_3$ at an activity of 2.0–2.5 μCi/ml. Vials were sealed with slotted stoppers to exclude air (and thus prevent animals from crawling out of the medium). Dark control animals were incubated in foil-wrapped vials. After incubation, animals were washed three times in sea water and homogenized in cold 100% methanol. Chlorophyll was extracted by phase separation into diethyl ether and measured spectrophotometrically (Strain and Svec, 1966). The ether extract, examined for radioactivity by liquid scintillation counting (LSC), contained negligible activity. The remaining homogenate was centrifuged and a 0.1 ml subsample of the alcoholic/aqueous supernatant fluid was removed for LSC. The centrifuged pellet was suspended in 1 ml tissue solubilizer for 24 h, and 0.1 ml of the solubilized fraction was removed for LSC. CPM rates were corrected for counting efficiency, background, quenching, and dilution factors, and uptake rates calculated as $DPM \cdot \mu g$ $chlorophyll^{-1} \cdot h$.

Chlorophyll retention

Animals were removed from stock cultures, placed in a newly-prepared (alga-free) aquarium, and maintained as described above. Animals withdrawn at intervals were relaxed in 8% $MgCl_2 \cdot 6H_2O$ and measured. After a recovery period in seawater, carbon fixation rates and chlorophyll levels were estimated as described above. Chlorophyll concentrations were calculated as milligrams per gram dry weight of animal tissue, using an empirically derived equation for animal weight: $Log_{10}W = 3.166\ Log_{10}L + 0.928$ ($r = .94$), where W = dry weight in micrograms and, L = relaxed length in millimeters.

Effect of temperature on fixation rate

Fixation rates were measured over a range of 15–35°C, corresponding to the temperature range measured at the Key Largo habitat over 5 years. Animals were acclimated at the test temperature for 24 h before isotope incubation (irradiation rate 325 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$.) Six animals were used for each light incubation and for each dark control.

Total carbonate content of seawater was measured by the acidometric method (Strickland and Parsons, 1972), and carbon fixation rates were estimated from the equation (after Colijn and Van Buurt, 1975): $\mu g\ C\ fixed = (A_l - A_d)/(A_a) \times C \times 1.06$ where A_l = light fixation activity (DPM), A_d = dark fixation activity, A_a = activity added to sample, C = total carbon available in incubation medium; and 1.06 = isotope discrimination ratio.

Effect of irradiation rate on fixation rate

Animals were incubated at varied light intensities at 25°C, using layers of gray fiberglass window screening as neutral density filters. Incandescent lamps were used for irradiation rates of 25–100$\cdot m^{-2} \cdot s^{-1}$; sunlight was used for higher rates.

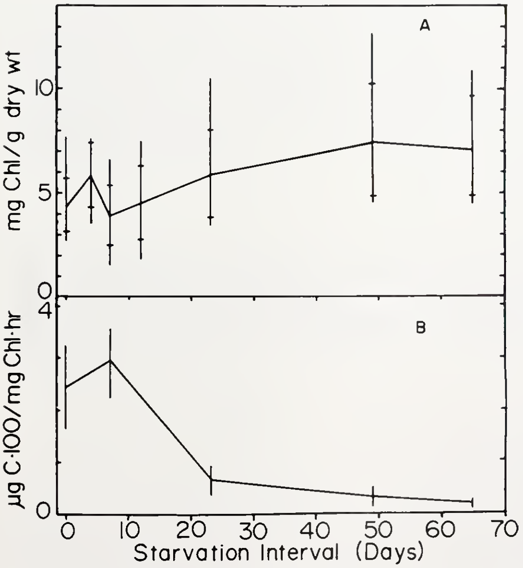
RESULTS

Starvation

Costasiella lilianae retained photosynthetically active chloroplasts, as evidenced by significant carbon fixation in both freshly fed and starved animals (Fig. 1). Chlorophyll levels increased somewhat after long-term starvation (65 days), but carbon fixation rate gradually declined after starvation periods longer than a week. Light fixation was significantly higher than dark fixation ($p < 0.05$) even after 65 days starvation (Fig. 1).

Temperature effects

Carbon fixation by *C. lilianae* varied widely as a function of temperature (Figs. 2, 3). Net (light minus dark) fixation increased nearly threefold from 20° to 30°C but declined somewhat at 35°C. Alcohol soluble (AS) and alcohol insoluble (AI) fraction were differentially affected by temperature: at suboptimal temperatures, most of the total fixation is in the AS fraction, but nearly equal levels of AI and AS components were fixed at temperatures optimal for total fixation. These frac-



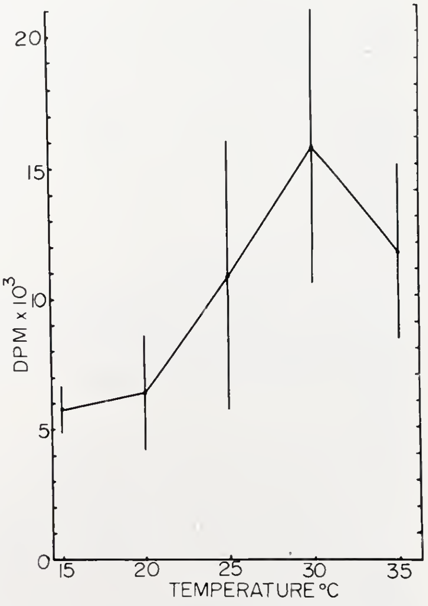
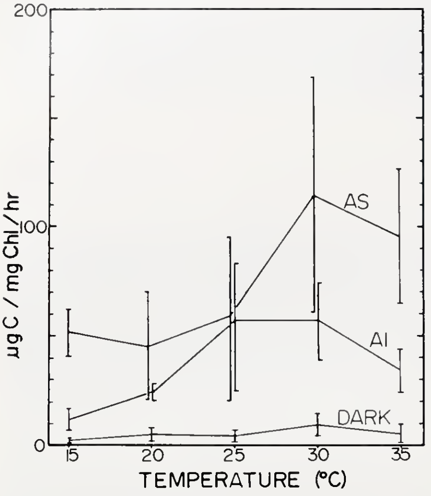


FIGURE 1. (Left) A. Effect of starvation on chlorophyll level (mean, standard deviation, and range shown; n = 10). B. Effect of starvation on net fixation rate (mean ± standard deviation, n = 10).

FIGURE 2. (Right) Effect of temperature on net total (alcohol soluble ± alcohol insoluble) carbon fixation by *Costasiella lilianae* (mean ± standard deviation; n = 6).



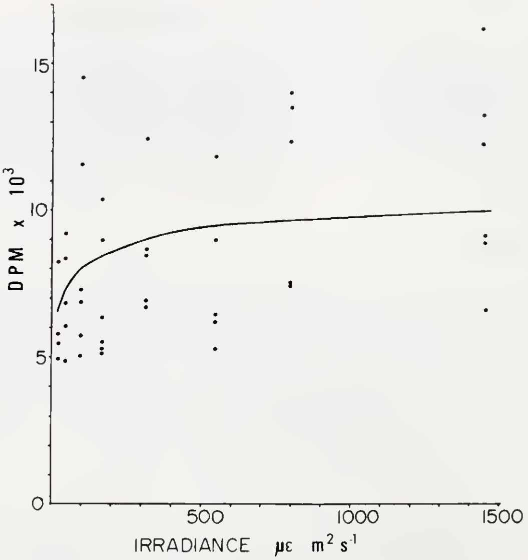


FIGURE 3. (Left) Effect of temperature on net alcohol-soluble (AS), net alcohol insoluble (AI) and dark (AI + AS) carbon fixation by *Costasiella lilianae*. (mean ± SD, n = 6).

FIGURE 4. (Right) Effect of light intensity on carbon fixation rate in *Costasiella lilianae*. Regression line calculated for interval of 25–15,000 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$ by method of least squares with semilogarithmic transformation: $y = 2608 \log x + 2750$, where x = DPM, y = intensity; $r = .47$. Relationship highly significant at $p < .01$.

tions are believed to represent short chain (AS) and long chain (AI) photosynthetic products (Grant *et al.*, 1976; Howard *et al.*, 1977).

Long-chain fractions are presumably used for structural synthesis (Grant *et al.*, 1976; Howard *et al.*, 1977; Stirts and Clark, 1980). Hence, diversion of photosynthates to somatic growth and gametogenesis is probably temperature dependent. Stirts and Clark (1980) noted similar thermal effects for *Elysia tuca* Marcus and Marcus. Dark fixation did not vary significantly with temperature (Fig. 3).

Light intensity effects

Carbon fixation approached saturation at fairly low irradiance levels, about 500 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$, a third of peak *in situ* levels (Fig. 4). Fixation was substantial even at low intensities (25 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$), suggesting that light intensity is a minor limiting factor under natural conditions. No inhibition was noted at the highest irradiance levels.

Digestive gland anatomy and fine structure

A single duct of the digestive gland extended into each ceras (Fig. 5A) where it branched into a grape-like cluster of sacs. The diverticular sacs were lined by cells (Type 1) 20–30 μm wide, containing the plastids (Fig. 5B). Light microscopy showed the plastids as 3–7 μm bodies containing a single dark-staining pyrenoid. A second cell type (Type 2) lacked plastids and had a granular cytoplasm.

Electron micrographs of digestive gland diverticula of unstarved animals (Fig. 6) also showed the two cell types. Type 1 cells contained abundant chloroplasts 3–

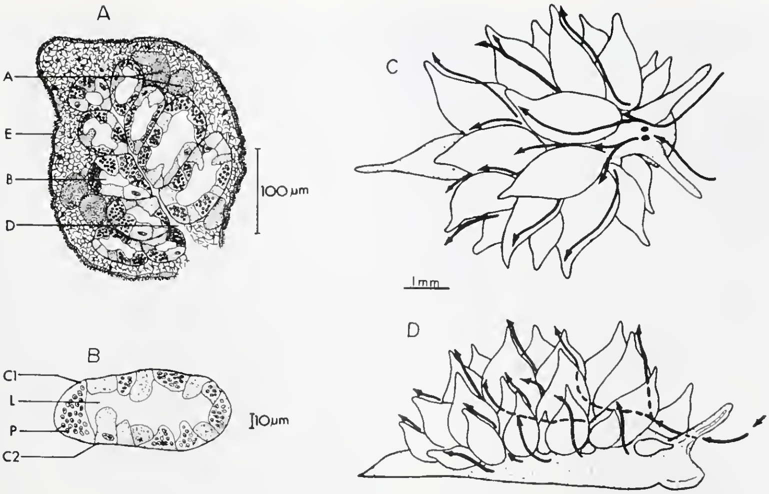


FIGURE 5. A. Sagittal section through ceras of *Costasiella lilianae*. B. Enlargement of single digestive diverticular pouch. C. Dorsal view of animal showing ciliary currents. D. Right lateral view. Abbreviations: A—Albumen gland; B—Lumen of diverticulum; C1—Type 1 cell; C2—Type 2 cell; D—Digestive gland duct; E—ciliated epithelium; L—Lumen of diverticulum; P—plastids.

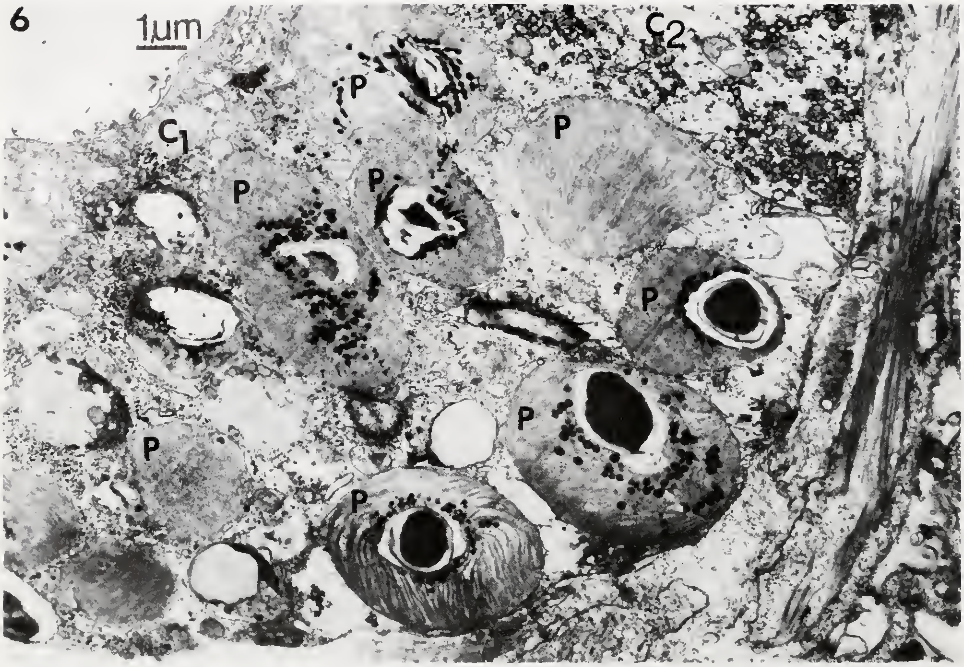
7 µm long, with abundant thylakoids and plastoglobuli, and a large (1–1.5 µm) central pyrenoid composed of an electron-dense core surrounded by a starch ring. The structure of the chloroplast was virtually identical to *Avrainvillea* plastids described by Hori and Ueda (1967). The chloroplast was bounded by an intact organellar membrane, and sometimes enclosed by a host membrane. Some plastids had regular elliptical outline, and others were irregular, possibly due to differences in the plane of sectioning with respect to the axis of the chloroplast. Many plastids contained a small bleb of granular material adherent to the outer surface and enclosed within the plastid membrane (Fig. 9). This material appeared to contain ribosomes and small vacuoles. Type 2 cells lacked plastids and highly vacuolated granular cytoplasm.

Plastid degeneration in *C. lilianae* is similar to that of other ascoglossans (Trench *et al.*, 1973; Trench, 1975; Switzer-Dunlap, 1975). It involves changes in electron density of plastids and formation of interthylakoid vesicles. Plastids within single cells are fairly uniform in state of decomposition. This suggests that a cell may phagocytize the entire complement of plastids during a specific period in maturation of cerata. Marcus and Marcus (1969) noted that the largest cerata sometimes contained degenerate symbionts (originally described as "zooxanthellae").

In animals starved for 2 weeks, some Type 1 cells contained intact plastids, but others contained plastids with early degenerative changes (Figs. 8, 9). These plastids were more electron dense than fresh plastids, and some showed early stages of thylakoid delamination and interthylakoid vesicle formation. As in unstarved animals, host membranes surround some plastids, but not others. The presence or absence of host membranes did not seem to correlate with plastid condition. Some host membranes appeared to form a continuous envelope around the plastid, while membranes formed from several vesicles enclose other plastids (Figs. 7, 8).

In animals starved for 4 weeks, some cells show further plastid degeneration (Fig. 10), though other cells contained intact plastids. Degenerating plastids had

6



7

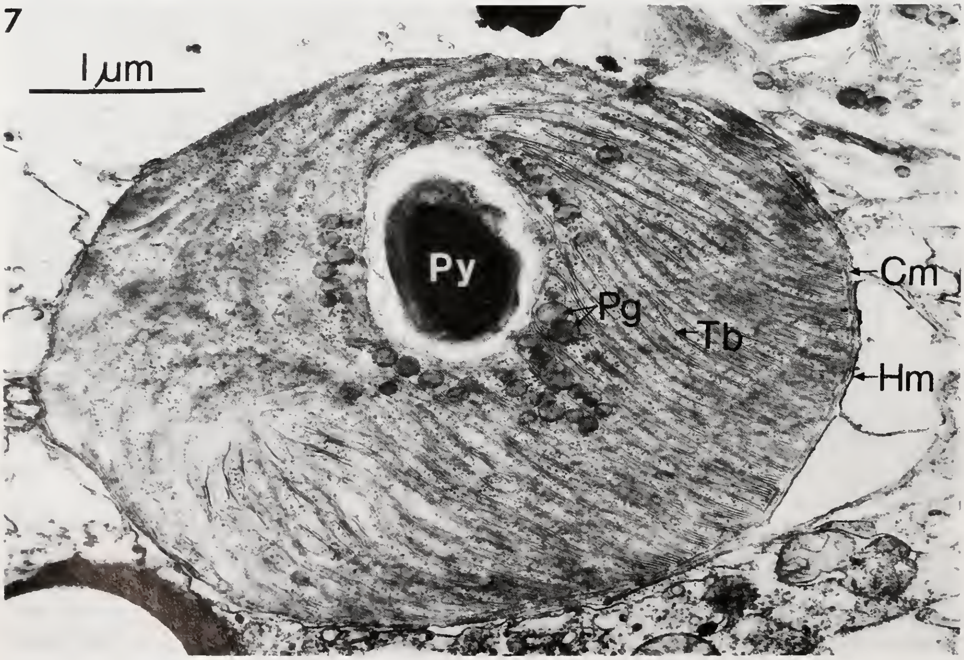
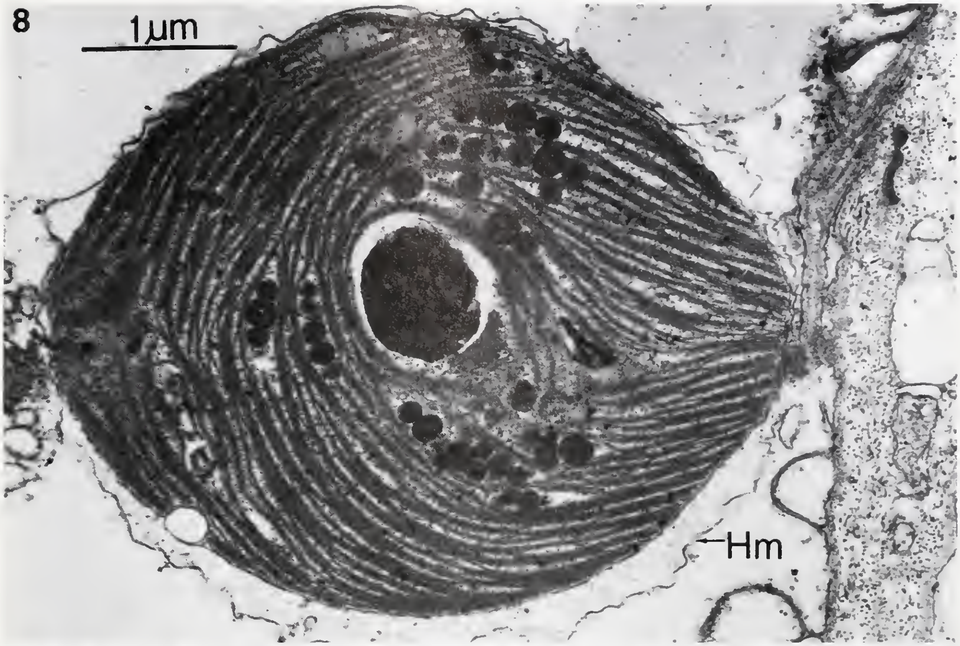


FIGURE 6. Section through diverticulum of digestive gland of unstarved *Costasiella lilianae*. C_1—type 1 cell; C_2—type 2 cell; P—plastid.

FIGURE 7. Symbiotic chloroplast from digestive gland cell of unstarved *Costasiella lilianae*. Cm—chloroplast membrane; Hm—host membrane; Pg—plastoglobuli; Py—pyrenoid; Tb—Thylakoid band.

8



9

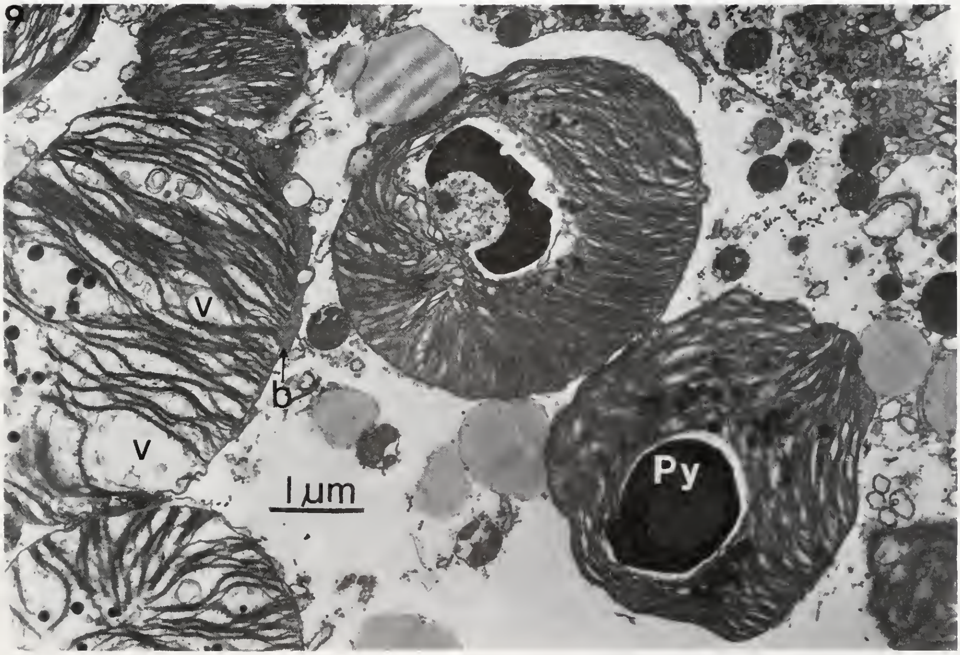


FIGURE 8. Intact chloroplast from digestive gland cell of 2-week-starved *C. lilianae*. Hm—host membrane.

FIGURE 9. Chloroplasts from digestive gland cell of 2-week-starved *C. lilianae*, showing early degenerative changes. b—cytoplasmic bleb enclosed within plastid membranes; Py—pyrenoid; v—interthylakoid vesicle.

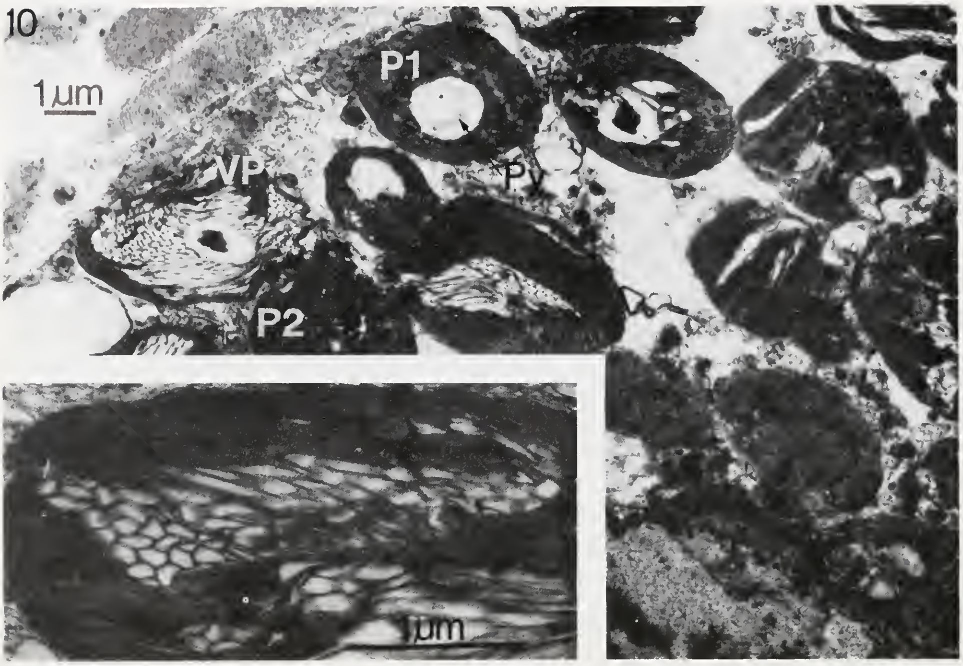


FIGURE 10. Digestive gland cell from 4-week-starved *C. lilianae*. P1, P2—electron dense plastids; Py—pyrenoid (note low density); VP—vesiculated plastid; inset—enlargement of vesiculated plastid.

highly vesicularized plastids, though outer membranes were still intact and thylakoids were recognizable in some plastids. Thylakoids were electron dense, plastoglobuli were indistinct, and some pyrenoids were electron transparent. Vacuoles enclosed most plastids. The cytoplasm contained numerous electron-dense bodies, somewhat smaller than intact plastids. These may have been final stages of plastid degeneration.

Functional morphology

Abundant saccate digestive diverticula almost completely fill the cerata and tail of *C. lilianae*. A pericardial vein complex occurs at the bases of the cerata (not shown). A dense layer of cilia covers the ceratal surfaces. These cilia create a strong circulation over ceratal surfaces and the pericardial network (Fig. 5C, D). This morphology differs from the flattened parapodial arrangement of the elysiids, but is functionally similar. The internal branching of the diverticula creates a large area for storage of plastids, and the cerata provide a large area for light absorption and gas exchange. The arrangement of cerata, cilia, and pericardial veins probably functions as a "negative gill" (for uptake of CO_2 and release of O_2), as in the elysiids (Stirts and Clark, 1980).

These apparent adaptations for gas exchange suggest that CO_2 transport strongly limits symbiotic photosynthesis. The effect of light on photosynthetic rate (Fig. 4) was rather weak at all but the lowest irradiance rates, suggesting that some other factor is limiting. The irradiance effect was measured near the thermal optimum, so CO_2 is the most probable limiting factor. Under optimal conditions, about 0.3–0.9 μg C was fixed per incubation vial, about 1% of the total CO_2 available. This is enough to noticeably raise pH (Sverdrup *et al.*, 1942), possibly removing CO_2 by carbonate precipitation. This would be an artifact of small experimental water volume, but similar effects in host tissues may explain the "lime spherules" in digestive gland cells of plastid-retentive species (Kawaguti and Yamasu, 1965; Taylor, 1968; Switzer-Dunlap, 1975; Graves *et al.*, 1979). These "lime spherules" are apparently absent in aposymbiotic species (Graves *et al.*, 1979).

DISCUSSION

Chlorophyll retention time, carbon fixation rate, structural integrity of chloroplasts, and stability of photosynthetic efficiency of *C. lilianae* are qualitatively and quantitatively equivalent to those of the most efficient elysiids. *Elysia viridis* (Montagu) and *Tridachia crispata* Mörch retain plastids up to 3 months (Trench and Ohlhorst, 1976), but retention varies with maintenance conditions. Chlorophyll level in *T. crispata* decreased 80% in 16 days (Clark and Busacca, 1978) under the same conditions as the present study. Retention in *E. viridis* is temperature dependent, with a Q_{10} of about 2 (calculated from data of Hinde and Smith, 1975) to 3.4 (from data of Trench *et al.*, 1973). Switzer-Dunlap (1975) found that light quality affected plastid retention in *Plakobranchus ocellatus* (van Hasselt). Another possible influence is the plastid source and its natural plastid turnover rate; algae with rapid plastid turnover may provide plastids that hosts do not retain long. This might explain variability in *T. crispata*, which feeds on and retains plastids from a variety of Chlorophyta (Clark and Busacca, 1978; Jensen, in press).

Few data are available for interfamilial comparison of photosynthetic efficiencies (Table I). Light:dark fixation ratios vary widely, and must be interpreted with caution, because light fixation rates may be measured at suboptimal conditions.

TABLE I

Comparison of light:dark fixation ratios and net carbon fixation rates in ascoglossan molluscs.

Species	Family	Light/dark ratio	Net fixation (μgC·mg $Chl^{-1}\cdot hr^{-1}$)	References
Costasiella lilianae (Marcus)	Hermaeidae	18–90	200–300	Present study
Limapontia depressa A & H	Stiligeridae	9.5		Hinde & Smith, 1974
Elysia viridis (Montagu)	Elysiidae	33	95	Trench *et al.*, 1973 Hinde & Smith, 1974, 1975
Elysia hedgepethi Marcus	Elysiidae	9.0		Greene & Muscatine, 1972
Elysia tuca Marcus	Elysiidae	100	60	Goetzfried, 1977 Stirts & Clark, 1980
Elysia cauze Marcus	Elysiidae	22		Clark *et al.*, 1979
Plakobranchus ocellatus van Hasselt	Elysiidae	6.3		Greene & Muscatine, 1972

Carbon mass fixation rates are available only for three species (Table I). Maximum rates for *C. lilianae* are 2 to 6 times those of elysiid species.

Long-term, highly efficient chloroplast symbiosis in a ceratiform ascoglossan clearly shows that the symbiosis is not limited to the Elysiidae as previously proposed (Muscatine and Greene, 1973; Hinde and Smith, 1974; Trench, 1975; Muscatine *et al.*, 1975; Graves *et al.*, 1979). Other less pronounced and possible examples of stiligeroid symbioses were summarized by Clark and Busacca (1978) (also see Taylor, 1967; Kremer and Schmitz, 1976).

If plastid symbioses occur within stiligeroid families as well as in Elysiidae, some may also occur among the common ancestors of the two evolutionary lines, the tectibranch Ascoglossa. Several tectibranchs have not shown functional plastids (Muscatine and Greene, 1973; Clark *et al.*, unpublished data), but some evidence of functional plastids exists (Stirts, 1980; Clark, in press).

Poorly understood factors affect plastid maintenance. Symbiotic capacity varies within populations, as in *Limapontia depressa* (Alder and Hancock) (see Hinde and Smith, 1974). Fixation rates also vary widely within populations. Coefficients of variation in fixation rate range from 0.16 to 0.65 in this study, and from 0.35 to 0.41 for two elysiids (Greene and Muscatine, 1972; our calculations).

Several major factors probably determine whether an ascoglossan retains chloroplasts as symbionts. Diet appears to be one such factor, as only certain types of algal plastids seem to be retained as symbionts. Siphonalean plastids resist treatments which destroy other plastids (Giles and Sarafis, 1974), and hence may resist digestion by host cells (Trench *et al.*, 1973; Trench, 1975; Gallop *et al.*, 1980). However, this cannot be the only factor, because many tectibranch species are aposymbiotic despite siphonalean diet (Muscatine and Greene, 1973), and nonsiphonalean plastids support well-developed symbioses (Taylor, 1971; Hinde and Smith, 1974; Graves *et al.*, 1979). Variable melanism, influenced by diet, is common among stiligerids (see Clark, 1975); the results of Hinde and Smith (1974) suggest that melanism is associated with non-functional plastids. Other factors may relate to cost-benefit aspects of symbiosis—whether photosynthetic yield to the host exceeds costs of plastid maintenance. Rates at which substances "leak" from plastids may also affect net benefit to the host (see Gallop, 1974). Climatic factors, especially temperature and light, obviously are important determinants of photosynthetic yield (Hinde and Smith, 1975; Goetzfried, 1977; Clark *et al.*, 1979; Stirts and Clark, 1980; present study). Intersections of these several "sets" of factors may greatly reduce the total number of symbiotic Ascoglossa, and give a false impression of disjunct distribution of symbiosis when few species have been examined. However, there does not appear to be a major phylogenetic determinant of symbiotic capacity within the non-tectibranch Ascoglossa.

ACKNOWLEDGMENTS

This research was supported by the National Science Foundation Grants DEB-7815449 and SER77-06567, and by a Sigma Xi Grant-in-aid to H. M. Stirts. We thank A. Goetzfried for technical assistance and Dr. G. N. Wells for technical advice.

LITERATURE CITED

CLARK, K. B. 1975. Nudibranch life cycles in the northwest Atlantic and their relationship to the ecology of fouling communities. *Helgol. Wiss. Meeresunters.* **27:** 28–69.

CLARK, K. B. In press. A new *Volvatella* from Bermuda. *Bull. Mar. Sci.* **31.**

CLARK, K. B., AND M. BUSACCA. 1978. Feeding specificity and chloroplast retention in four tropical Ascoglossa, with a discussion of the extent of chloroplast symbiosis and the evolution of the order. *J. Molluscan Stud.* **44:** 272–282.

CLARK, K. B., M. BUSACCA, AND H. STIRTS. 1979. Nutritional aspects of development of the ascoglossan *Elysia cauze*. Pp. 11–24 in S. E. Stancyk, Ed., *Reproductive Ecology of Marine Invertebrates.* Univ. of South Carolina Press, Columbia, S. C.

COLIJN, R., AND G. VAN BUURT. 1975. Influence of light and temperature on the photosynthetic rate of marine benthic diatoms. *Mar. Biol.* **31:** 209–214.

GALLOP, A. 1974. Evidence for the presence of a 'factor' in *Elysia viridis* which stimulates photosynthate release from its symbiotic chloroplasts. *New Phytol.* **83:** 445–450.

GALLOP, A., J. BARTROP, AND D. C. SMITH. 1980. The biology of chloroplast acquisition by *Elysia viridis. Proc. R. Soc. Lond. B. Biol. Sci.* **207:** 335–349.

GILES, K. L., AND V. SARAFIS. 1974. Implications of rigescent integuments as a new structural feature of some algal chloroplasts. *Nature* **248:** 512–513.

GOETZFRIED, A. 1977. A comparison of light dependence of photosynthesis in a sacoglossan *Elysia tuca* and its algal food *Halimeda discoidea. M.S. Thesis, Florida Inst. of Technol.*, Melbourne. 43 pp.

GRANT, B. R., R. S. HOWARD, AND K. R. GAYLOR. 1976. Isolation and properties of chloroplasts from the siphonous green alga *Caulerpa simpliciuscula. Aust. J. Pl. Physiol.* **3:** 639–651.

GRAVES, D. A., M. A. GIBSON, AND J. S. BLEAKNEY. 1979. The digestive diverticula of *Alderia modesta* and *Elysia chlorotica* (Opisthobranchia:Sacoglossa). *Veliger* **21:** 415–422.

GREENE, R. W. 1970. Symbiosis in sacoglossan opisthobranchs: symbiosis with algal chloroplasts. *Malacologia* **10:** 357–368.

GREENE, R. W., AND L. MUSCATINE. 1972. Symbiosis in sacoglossan opisthobranchs: photosynthetic products of animal-chloroplast associations. *Mar. Biol.* **14:** 253–259.

HINDE, R., AND D. C. SMITH. 1974. Chloroplast symbiosis and the extent to which it occurs in Sacoglossa (Gastropoda:Mollusca). *Biol. J. Linn. Soc.* **6:** 349–356.

HINDE, R., AND D. C. SMITH. 1975. Role of photosynthesis in the nutrition of the mollusk *Elysia viridis. Biol. J. Linn. Soc.* **7:** 161–171.

HORI, T., AND R. UEDA. 1967. Electron microscope studies on the fine structure of plastids in siphonous green algae with special reference to their phylogenetic relationships. *Sci. Rep. Tokyo Kyoiku Daigaku Sec. B.* **12:** 225–244.

HOWARD, R. S., B. R. GRAND, AND H. FOCK. 1977. Storage and structural products formed during photosynthesis in the siphonous alga *Caulerpa simpliciuscula* (Chlorophyceae). *J. Phycol.* **13:** 340–345.

JENSEN, K. R. In press. A review of sacoglossan diets, with comparative notes on radular and buccal anatomy. *Malacol. Rev.* **14.**

KAWAGUTI, S. AND T. YAMASU. 1965. Electron microscopy on the symbiosis between an elysioid gastropod and chloroplasts from a green alga. *Biol. J. Okayama Univ.* **11:** 57–65.

KREMER, B. P., AND K. SCHMITZ. 1976. Aspects of $^{14}CO_2$-fixation by endosymbiotic rhodoplasts in the marine opisthobranchiate *Hermaea bifida. Mar. Biol.* **34:** 313–316.

MARCUS, E., AND E. MARCUS. 1969. Euthyneure Meeresschnecken Brasiliens (2). *Beitr. Neotrop. Fauna* **6:** 1–16.

MUSCATINE, L., AND R. W. GREENE. 1973. Chloroplasts and algae as symbionts in molluscs. *Int. Rev. Cytol.* **36:** 137–169.

MUSCATINE, L., R. R. POOL, AND R. K. TRENCH. 1975. Symbiosis of algae and invertebrates: aspects of the symbiont surface and the host-symbiont interface. *Trans. Amer. Microsc. Soc.* **94:** 450–469.

STIRTS, H. M. 1980. Chloroplast symbiosis in tectibranch ascoglossans (Opisthobranchia:gastropoda). *Ph.D. dissertation, Florida Institute of Technology*, Melbourne. 120 pp.

STIRTS, H. M., AND K. B. CLARK. 1980. Effects of temperature on products of symbiotic chloroplasts in *Elysia tuca* Marcus (Opisthobranchia:Ascoglossa). *J. Exp. Mar. Biol. Ecol.* **43:** 39–47.

STRAIN, H. H., AND W. A. SVEC. 1966. Extraction, separation, estimation, and isolation of the chlorophylls. Pp. 21–66 in L. P. Vernon and G. R. Sheely, Eds., *The Chlorophylls*, Academic Press, New York.

STRICKLAND, J. D. H., AND T. R. PARSONS. 1972. A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa, 310 pp.

SVERDRUP, H. U., M. W. JOHNSON, AND R. H. FLEMING. 1942. *The oceans: their physics, chemistry, and general biology*. Prentice-Hall, Englewood Cliffs, New Jersey. 1087 pp.

SWITZER-DUNLAP, M. 1975. Symbiosis between algal chloroplasts and the mollusc *Plakobranchus ocellatus* van Hassell (Sacoglossa:Opisthobranchia). *Ph.D. Dissertation, University of Hawaii*, Honolulu. 167 pp.

TAYLOR, D. L. 1967. The occurrence and significance of endosymbiotic chloroplasts in the digestive glands of herbivorous opisthobranchs. *J. Phycol.* **3:** 234–235.

TAYLOR, D. L. 1968. Chloroplasts as symbiotic organelles in the digestive gland of *Elysia viridis* (Gastropoda, Opisthobranchia). *J. Mar. Biol. Assoc. U. K.* **48:** 1–15.

TAYLOR, D. L. 1971. Symbiosis between the chloroplasts of *Griffithsia flosculosa* (Rhodophyta) and *Hermaea bifida* (Gastropoda:Opisthobranchia). *Pubbl. Staz. Zool. Napoli* **39:** 116–120.

TRENCH, R. K. 1969. Chloroplasts as endosymbiotic organelles in *Tridachia crispata* (Bergh), Sacoglossa, Opisthobranchia. Nature **222:** 1071–1072.

TRENCH, R. K. 1975. Of "leaves that crawl": functional chloroplasts in animal cells. *Symp. Soc. Exp. Biol.* **29:** 229–265.

TRENCH, R. K., J. E. BOYLE, AND D. C. SMITH. 1973. The association between chloroplasts of *Codium fragile* and the mollusc *Elysia viridis*. II. Chloroplast ultrastructure and carbon fixation in *E. viridis*. *Proc. R. Soc. Lond. B. Biol. Sci.* **184:** 63–81.

TRENCH, R. K., R. W. GREENE, AND B. G. BYSTROM. 1969. Chloroplasts as functional organelles in animal tissues. *J. Cell. Biol.* **42:** 404–417.

TRENCH, R. K. AND S. OHLHORST. 1976. The stability of chloroplasts from siphonaceous algae in symbiosis with sacoglossan molluscs. *New Phytol.* **76:** 99–109.

Reference: *Biol. Bull.*, **160:** 55–68. (February, 1981)

ACTION OF VARIOUS ANTICOAGULANTS ON HEMOLYMPHS OF LOBSTERS AND SPINY LOBSTERS

MICHÈLE DURLIAT AND ROGER VRANCKX

Biologie et Physiologie des Organismes Marins, Université P. et M. Curie, 4 place Jussieu, 75230 Paris CEDEX 05, France

ABSTRACT

Effects of anticoagulants on hemolymph coagulation of lobsters are reported. Effects of some inhibitors on plasma clotting change in relation to molting stages of animals. Variations of electrophoretic and immunochemical protein patterns are analyzed. Complete coagulation inhibition shows that the plasma coagulogen exists as a mixture of soluble uncovalently crosslinked polymers. In lobsters the molecular weight of the lightest visible circulating unit is approximately 420,000 daltons. The weight interval between each of the four lighter polymers is about 200,000 daltons. Serine protease inhibitors impair plasma clotting and interact with cellular clumping.

INTRODUCTION

Several types of coagulation have been described in Arthropoda hemolymph (review in Grégoire, 1971). In Crustacea, three types of clotting processes have been defined (Tait and Gunn, 1918): cellular clumping (type A), complete plasma gelation (type C), and intermediates between these two types (type B).

In type A, the hemolymph remains fluid and only cellular factors seem to be involved in the clumping. This has not been studied in Crustacea, although Levin (1967) demonstrated that some cellular extracts from *Maia squinado* and *Carcinus maenas* clot on addition of endotoxin as cell lysates of *Limulus* do. In horseshoe crabs, the soluble coagulogen is contained within the hemocytes (Mürer *et al.*, 1975; Levin and Bang, 1968; Solum, 1970; Shishikura *et al.*, 1977). It is characterized by a small molecular weight and is converted into an insoluble coagulin by a serine protease enzyme localized in amoebocytes (Young *et al.*, 1972; Solum, 1973; Sullivan and Watson, 1975; Tai and Liu, 1977; Shishikura and Sekiguchi, 1978). The resulting clot is stabilized by noncovalent crosslinks (Tai *et al.*, 1977; Mosesson *et al.*, 1979).

In type C, the clottable factor ("fibrinogen") is a plasma protein. It has been studied in *Homarus* (Glavind, 1948; Duchateau and Florkin, 1954; Doolittle and Lorand, 1962; Stewart *et al.*, 1966), in *Panulirus interruptus* (Tyler and Scheer, 1945; Fuller and Doolittle, 1971a, b; Doolittle and Fuller, 1972) and in *Astacus leptodactylus* (Durliat and Vranckx, 1976). It has a high molecular weight and can be converted to a gel which seems to be crosslinked covalently by the action of a cellular transglutaminase (Bruner-Lorand *et al.*, 1966; Myhrman and Bruner-Lorand, 1970). Plasma factors of vertebrate blood have no effect on hemolymph gelation. Moreover, neither proteolysis nor fibrinolysis has been demonstrated in

Received 6 February 1980, accepted 7 October 1980.

Abbreviations: NEM, N-ethyl maleimide; PCMB, parachloromercuribenzoate; PMSF, phenylmethane sulfonyl fluoride; DIFP, diisopropyl fluorophosphate; GME, glycine methyl ester; and DC, monodansylcadaverine.

Crustacea (Hawkey, 1970). These reports have led some authors to conclude that this plasma clotting has one step only: A soluble fibrinogen is transformed into an insoluble "fibrin-like" gel, (Fuller and Doolittle, 1971a, b) or more likely into a "fibrinogen-like" gel, by polymerization of a basic unit:

$$\text{n fibrinogen} \xrightarrow[\text{Ca}^{2+}]{\text{transamidase}} \text{cross-linked (fibrinogen) n-gel (Lorand, 1972).}$$

In fact, a cellular clumping appears in each type of coagulation. A process similar to that in *Limulus* might exist in Crustacea either at the cellular level alone or also at an early step in plasma clotting.

This study reports our attempts to prevent both plasma clotting and cellular clumping in lobster and spiny lobster hemolymphs by inhibitors of enzymes involved in the two types of clotting processes, and also to determine whether these two phenomena are interdependent, perhaps in a sequential manner.

MATERIALS AND METHODS

Homarus vulgaris lobsters were a gift of "La Langouste" fisheries (Roscoff, France). Spiny lobsters (*Jasus lalendei, Jasus paulensis, Jasus frontalis, Panulirus regius, Palinurus vulgaris*) were obtained from local fish merchants. Molt stages were determined by examining uropods according to the method of Vranckx and Durliat (1978).

Serum and plasma were obtained from animals of both sexes in C3, C4, and premolt stages. Hemolymph was withdrawn from the periopod sinus and distributed into tubes. Sera were obtained according to a method previously described (Durliat and Vranckx, 1976). To delay or prevent coagulation, blood withdrawal was performed with a syringe containing a volume of chemicals identical to that of the hemolymph removed. Distilled water and sodium chloride solutions, at 1% or 3% (initial concentrations), were used to test the effects of dilution and osmolarity on clotting. Tests were also carried out in the presence of a 10% $CaCl_2$ solution. Three groups of anticoagulants, purchased from Sigma, were utilized in both hypotonic and isotonic media at the following initial concentrations:

1. As enzymatic inhibitors, we successively used: 10% sodium citrate or 5% EDTA solutions; 2.10^{-2} *M* NEM (N-ethyl-maleimide) with or without sodium citrate; 2.10^{-2} *M* PCMB (parachloromercuribenzoate); 2.10^{-2} *M* PMSF (phenylmethane sulfonyl fluoride) or DIFP (diisopropyl fluorophosphate) dissolved in 5% 2-propanol, or 10% soy bean trypsin inhibitor.

2. Among amines which inhibit transglutaminase-catalysed crosslinking of proteins, GME (Glycine methyl ester) (10^{-2} *M* or 10^{-1} *M*) and DC (monodansylcadaverine) (10^{-3} *M*), dissolved in 5% acetone, were tested.

3. As an inhibitor of membrane disruption, we used propanolol. It preserved the membrane of granules without preventing the disruption of hemocytes (Mürer *et al.*, 1975).

All experiments were performed at 4°C. Alteration of hemocytes was observed through an optical microscope. One aliquot of hemolymph was centrifuged immediately at 1000 rpm for 5 min and the plasma was separated from hemocytes. The other portion was left undisturbed. Each sample was examined as soon as possible and again after storage (one month at −30°C). Uncytolysed hemocytes quickly separated from plasma were treated with distilled water, HCl, and NaOH, as described by Solum (1973).

Protamine sulfate gelation tests were carried out as described by Gurewich and Hutchinson (1971).

Electrophoretic analyses were performed on polyacrylamide gradient gels (4–30% and 2–16%) in 0.15 *M* Tris-borate buffer at pH 8.2 for 24 h at 50 V, or at pH 9.6 with approximately 100 V for 48 h. A protein calibration kit (Pharmacia) was used to estimate molecular weights.

We injected rabbits with pooled whole hemolymphs (citrated plasma and cell lysates) from lobsters of both sexes in premolt stages and also with pools of hemolymphs from spiny lobsters corresponding to different molting stages, according to previously published procedures (Durliat and Vranckx, 1976). All samples were tested against homologous anti-hemolymph sera in Tris barbital buffer of pH 8.6, μ:0.03, as given in the reference. Occasionally, line immunoelectrophoresis was also performed by the method of Krøll (1973).

RESULTS

Results of coagulation assays were nearly the same in lobsters and spiny lobsters, regardless of sex. Data are summarized in Table I.

In the absence of any anticoagulant, a cellular clump appeared. In freshly caught animals only, this clot then underwent a retraction. After a lapse of some minutes, the whole opaque hemolymph gelled and formed a firm transparent unretracted clot in the tube. In hypotonic medium, cellular lysis was very fast. Distilled water and 1% and 3% NaCl solutions had little effect on coagulation. Dilution slightly delayed clotting and produced a less firm gel. By contrast, with a 10% $CaCl_2$ solution, coagulation was even faster than in the reference sample.

TABLE I

Effects of anticoagulants on the hemolymph of Homarus vulgaris. *Clotting tests were performed on 20 lobsters. Intensity of responses is given by the following symbols: (−) represents no reaction; (+) is a weak reaction; (±) is a doubtful reaction; (++) is a moderate reaction, and (+++) is a strong reaction.*

Coagulation mechanisms Inhibitors	Cellular clump	Retraction of hemocyte clump	Plasma gelation
without	+++	+(+)	+++
distilled water	+++	++	++
NaCl 1%	++	+++	++
NaCl 3%	++	++	++
Sodium citrate, H_2O	+++	++	−
Sodium citrate, NaCl	++	−	−
NEM, H_2O	+(+)	−	−
NEM, NaCl	−	−	−
NEM, Sodium citrate, H_2O	+	−	−
NEM, Sodium citrate, NaCl	−	−	−
PSMF, H_2O	++	+	+
PSMF, NaCl	±	−	+
DIFP, H_2O	+	−	±
DIFP, NaCl	±	−	±
Trypsin inhibitor	±	−	−
NEM, PMSF, H_2O	+	−	−
NEM, PMSF, NaCl	−	−	−
Propanolol, NaCl	+	−	±
GME, H_2O	+++	+++	−
GME, NaCl	+++	+++	−
DC, H_2O	++	+	±
DC, NaCl	++	+	−

When blood samples were withdrawn with an anticoagulant in the isotonic medium, immediately centrifuged, and the cells discarded, no cellular phenomenon was observed and the plasma remained fluid.

When hemolymphs were not centrifuged, changes varied according to the inhibitor used. Sodium citrate and EDTA solutions did not inhibit cellular lysis but prevented plasma clotting. As NEM prevented cellular aggregation, the hemocytes sedimented but did not form networks or cellular clumps. This effect was more marked in the presence of Na and sodium citrate ions. The amoebocytes were preserved, and plasma did not coagulate. PMSF delayed gelling but did not inhibit plasma clotting, except in one animal in the C3 stage. Although the hemocytes were always quickly lysed, the clumping and its retraction were sometimes disturbed (in NaCl medium). Moreover, the coagulated plasma was soft and easily dissociated. DIFP and the trypsin inhibitor sometimes completely abolished or considerably delayed both plasma gelation and cellular clumping. Propanolol also delayed plasma gelation, which remained incomplete. The clotting time of *Homarus* plasma was considerably extended by amines that inhibit the crosslinking of vertebrate fibrin. GME and DC entirely inhibited plasma clotting but were without action on hemocytes, which formed a clot with a strong retraction of the coagulum.

The coagulated cell lysate was dissolved in 0.2 N HCl and then reprecipitated by neutralization with 0.2 N NaOH.

Principal data on electrophoretic analysis are presented in Tables II and III.

TABLE II

Effects of inhibitors on electrophoretical patterns of the hemolymph of Homarus vulgaris. *Experiments were performed on 10 lobsters. Supplementary bands are indicated by (a) fraction of about 420,000 daltons; (b) fraction with a molecular weight of approximately 200,000 daltons, which was only visible on gels running 48 h at pH 9.5 when the polymer scale was dissociated, and (c) aggregate with a high molecular weight. Symbols: (-), no reaction; (+), weak reaction; (±), doubtful reaction; (++), moderate reaction; (+++), strong reaction.*

Patterns Inhibitors	Polymer scale	Supplementary bands	Deposits on the top of gels	Plasmatic clotting
None	—	—		+++
Sodium citrate, H_2O	4–5 bands	a ±		–
Sodium citrate, NaCl				
NEM, H_2O	4–5 bands	—		–
NEM, NaCl				
NEM, Sodium citrate, H_2O	4–5 bands	a		–
NEM, Sodium citrate, NaCl				
NEM, Sodium citrate, NaCl centrifuged		b		
PMSF, H_2O	–	c	++	+
PMSF, NaCl	–	—		+++
PMSF, NaCl, centrifuged	–	b		+
DIFP, NaCl, centrifuged	–			++
NEM, PMSF, H_2O	5–6 bands	b	++	–
Propanolol, NaCl	–			++
Propanolol, NaCl centrifuged	7 bands			–
GME, 10^{-1} *M*	3–4 bands	a ± b		–
GME, 10^{-2} *M*, NaCl	8 bands	a + b		–
DC, H_2O	–	a + b		+
DC, H_2O, centrifuged	4–5 bands			–
DC, NaCl, centrifuged	7 bands	a + b		–

TABLE III

Effects of inhibitors on electrophoretical patterns of the Jasus lalendei *hemolymph. Experiments were carried out on 15 spiny lobsters. Additional bands are indicated by (a) fraction running in the area of 330,000 daltons; (b) fraction running slightly above the hemocyanin subunits in the area of 140,000 daltons; (c) aggregate with a high molecular weight, and (d) fraction running above 16 S hemocyanin. Other symbols: (—), no reaction; (+) weak reaction; (±), doubtful reaction; (++), moderate reaction; (+++), strong reaction.*

Patterns Inhibitors	Polymer scale	Additional bands	Plasmatic clotting
None	—	—	+++
Sodium citrate	5–6 bands	—	–
NEM	5–6 bands	a + b	–
NEM + EDTA	6–7 bands	2–3b 2d	–
PMSF, H_2O	—	c	++
PMSF, Propanol	—	b c	±
Propanol	—	b c	+
DIFP	5–6 bands	b	–
Trypsin inhibitor	5–6 bands	c	–
GME, 0.01 *M*	7–8 bands	d	–
GME, 0.1 *M*	5–6 bands	b d	–
DC	5–6 bands	b c	–
Triton	—	—	+++

In standard conditions (gels 4–30%, pH 8.2 and migration for 24 h at 50 V), *Homarus vulgaris* hemolymphs in the presence of any anticoagulant that inhibited plasma gelation showed a soluble polymer scale in addition to the serum pattern (Figs. 1a, b, c). According to the inhibitor used, this scale of polymers exhibited a more or less large number of bands in the different plasmas. These fractions represented the plasma clottable factor, since fluorescence appeared with DC above the oligomer of hemocyanin and up to the most cathodic fraction of high molecular weight. The number of polymers was especially high with saline solutions of propanolol after centrifugation and with diluted amines, *i.e.* with products which did not entirely inhibit this gelation. When GME (which like DC acted competitively) was more concentrated, the number of fractions decreased without going below four. With inhibitors of the crosslinking phase (sodium citrate or NEM), the freshly centrifuged samples showed 4–5 bands. These different scales were much more evident in 2–16% gels (Fig. 1d). Moreover, in samples that contained sodium citrate, NEM with sodium citrate, GME, or DC, one supplementary fraction appeared above the hemocyanin oligomer. In samples with PMSF H_2O, one aggregate of important molecular weight was present and some fractions did not enter the gel.

The estimated weights of the different scale units were 420,000; 670,000; 850,000; 1,050,000; and 1,200,000 (fluorescence of DC conjugates used as reference to clottable products). The main hemocyanin fraction was estimated to be 385,000 daltons. With dilute GME or DC, the progression between each additional band of the polymer scale was about 100,000 daltons.

However, when the running time of electrophoresis was lengthened so that the proteins could reach the "pore limit" of the gel, the different fractions of these scales began to smear (Figs. 1e, f). All the polymer scales disappeared when the experiments were carried out for 48 h at 100 V, with a buffer at pH 9.5, except in samples with DC or GME. Moreover, with centrifuged samples obtained in the

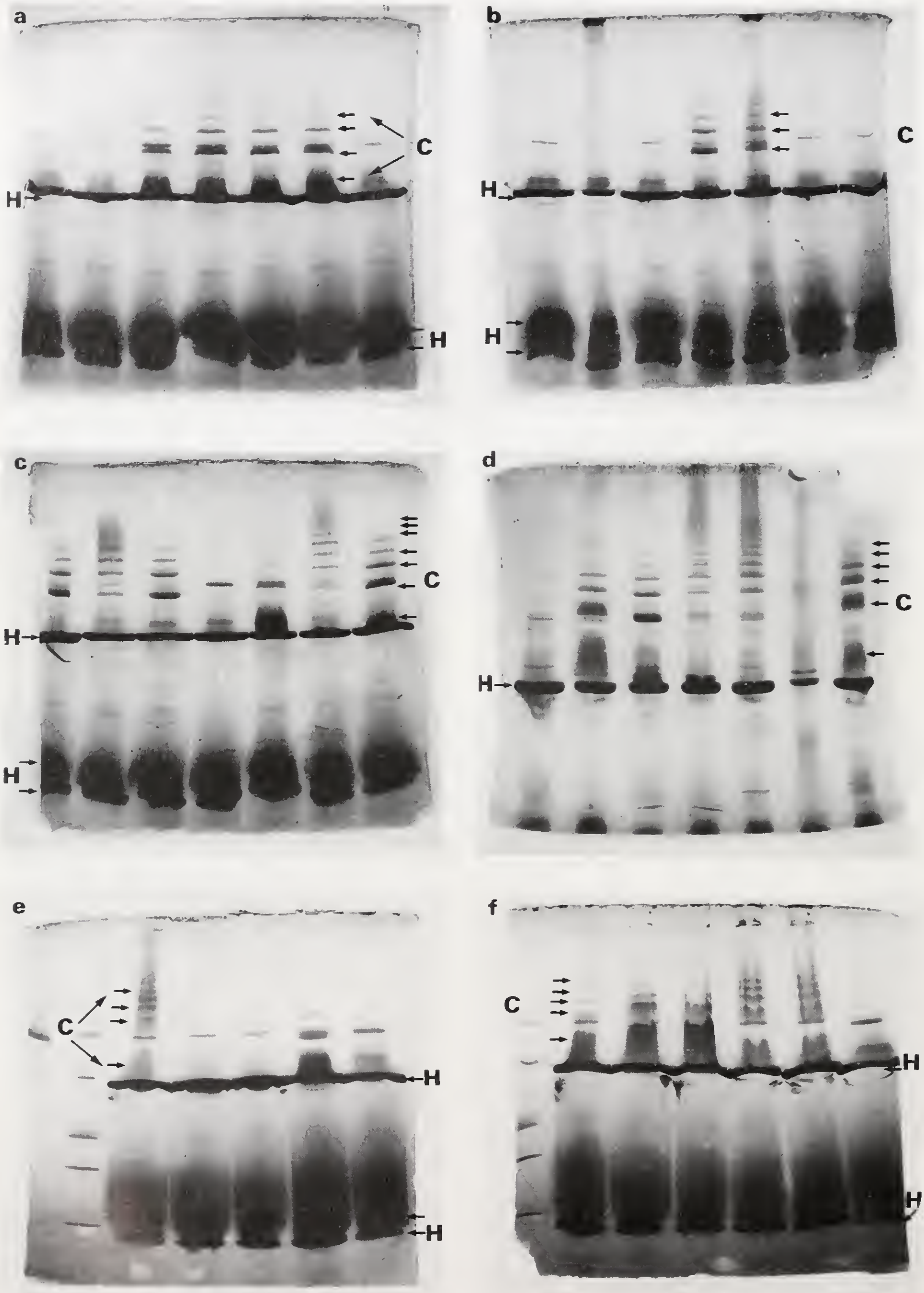


FIGURE 1. Effects of inhibitors on electrophoretic patterns of hemolymph from *Homarus vulgaris* on acrylamide gradient gels. (a), (b) and (c) show electrophoreses performed on 4–30% gels in standard conditions. (a) From left to right patterns of samples with NaCl 3%, propanolol, NEM sodium citrate H_2O, NEM sodium citrate NaCl, sodium citrate H_2O, sodium citrate NaCl, and serum. (b) From left

presence of saline solutions of NEM sodium citrate, PMSF, DC, or diluted GME, one band was more visible in the area of 200,000–250,000 daltons than with the other plasmas. Thus, fluorescence in the DC extract appeared in the scale of polymers but also in the area of 200,000–250,000 daltons as well as in that of 100,000 daltons.

When all blood samples, centrifuged or uncentrifuged, were submitted to electrophoresis after freezing storage, the number of polymers in the scale was higher in the uncentrifuged samples than in the centrifuged ones.

In spiny lobsters, electrophoresis was performed mainly on hemolymphs of *Jasus lalendei* and *Panulirus regius*. Data were similar to those reported in *Homarus* (Table III) but the plasma gel was firmer. One additional band was always present in the samples obtained with NEM, NEM in EDTA, or DC, and frequently with GME, PMSF (even after plasma clotting), or DIFP (Figs. 2a, b). This band ran slightly above the hemocyanin subunits, in the area of 140,000 daltons. This fraction was more easily visible when the hemocyanin oligomer was dissociated. The highest fraction of the polymer scale always appeared with PMSF or propanolol, but one intermediary band from this scale was absent; sometimes the same observations were noted either with a trypsin inhibitor or DC. Moreover, one or two additional fractions running between the hemocyanin hexamer and "fibrinogen" appeared in samples of GME or NEM in EDTA. In NEM one more easily visible fraction appeared in the area of 330,000 daltons (Figs. 2c, d).

In *Homarus vulgaris* a maximum of 13 precipitate lines was revealed by crossed immunoelectrophoresis (Fig. 3). Fractions present in all sera and plasmas were: 1 (hemocyanin), 3, 4, 8, and 10 (for identification of different fractions see Fig. 3). The presence or absence of other arcs depended on the inhibitors used, the experimental conditions, and on the sex and molting stages of the animals. All hemolymphs from female animals showed a group of cathodic precipitate lines (group 11), built up from two or three cross-reacting fractions, which did not appear in hemolymphs from male animals. In most experiments the thin cathodic fraction 12 was evident with whole hemolymphs but not with plasmas separated from uncytolysed amoebocytes. It was probably of cellular origin. Other proteins seemed to depend on the anticoagulant used. With DC a faint cathodic line 13 was shown and did not seem to relate to group 12, since it was present in the centrifuged sample. Two other fractions sometimes occurred both in sera and in plasmas: the very thin precipitate line 9, whose variations were not easily distinguishable, and the fraction 2, which moved into agarose as the hemocyanin did. These proteins may not have been connected with the clotting process, since they were absent in certain animals. When plasma gelation was inhibited, the soluble fibrinogen-like factor was always evidenced by a single precipitate line, 5 (Fig. 4), but the relative mobility of this factor 5 versus 4 varied according to the inhibitor used. Further-

---

to right patterns of samples with PMSF NaCl, PMSF H_2O, centrifuged PMSF NaCl, NEM H_2O, NEM-PMSF H_2O, NaCl, and serum. (c) From left to right patterns of hemolymphs with DC, propanolol NaCl, NEM sodium citrate NaCl, H_2O, concentrated GME-NaCl, and GME H_2O. (d) Electrophoresis carried out on 2–16% gradient gels. From left to right patterns of serum and plasmas with sodium citrate, NEM sodium citrate, GME-NaCl, propanolol-NaCl, NEM-PMSF-H_2O, and centrifuged DC. (e) and (f) Electrophoreses performed on 4–30% gels with a high pH buffer. (e) From left to right: molecular weight standards, centrifuged plasmas with saline solutions of DC, DIFP, PMSF, NEM sodium citrate, and serum. (f) From left to right: molecular weight standards, plasmas with EDTA, concentrated GME after and without centrifugation, diluted GME after and without centrifugation, and serum. H shows the hemocyanin and C represents the different fractions of the clottable factor.

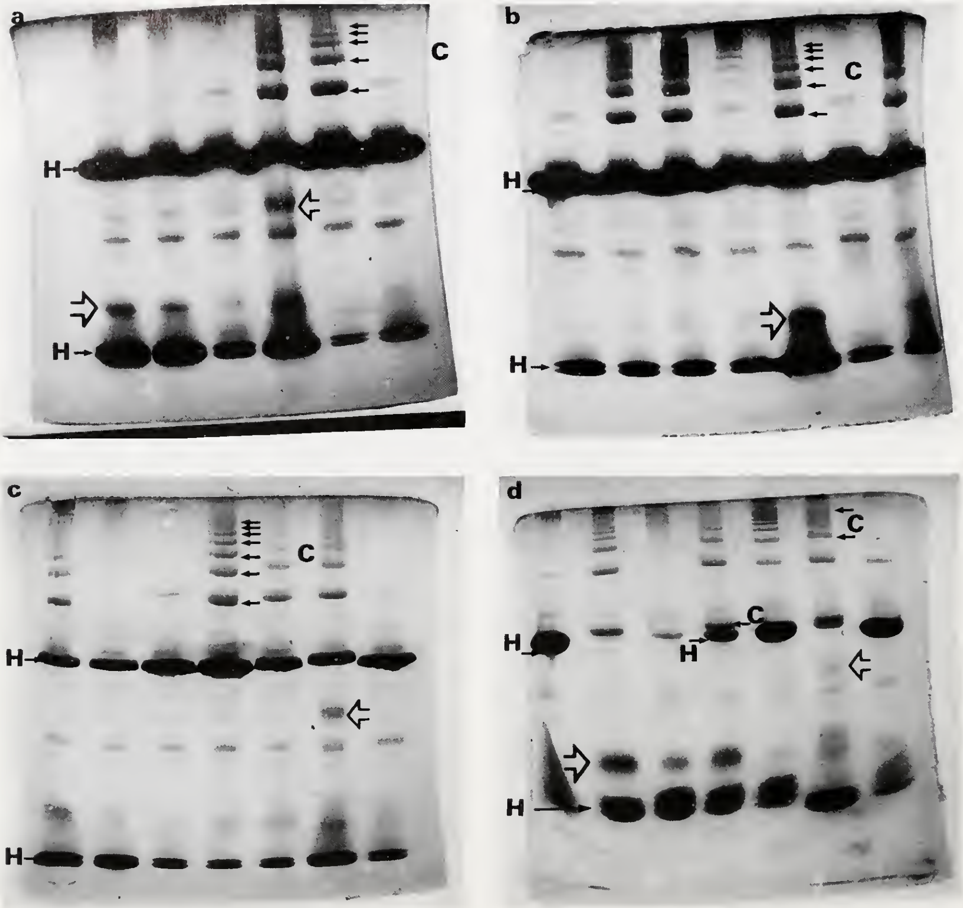


FIGURE 2. Effects of inhibitors on electrophoretical patterns of hemolymph from *Jasus lalendei* on acrylamide gradient gels (4–30%). (a) From left to right patterns of plasmas with propanolol, PMSF propanol, PMSF H_2O, NEM, sodium citrate, and serum. (b) From left to right patterns of serum, then samples obtained with concentrated and diluted GME, trypsin inhibitor, DC, triton, and NEM. (c) From left to right patterns of plasmas with DC, PMSF propanol, PMSF H_2O, diluted GME, concentrated GME, NEM, and serum. (d) From left to right patterns of plasmas with trypsin inhibitor, DC, PMSF propanol, GME, sodium citrate NEM, and serum. (a) and (b) Experiments were performed on animals in intermolt. (c) and (d) Experiments were carried out on animals in postmolt stage. The arrows show the additional fractions. H represents hemocyanin; C represents the different fractions of the clottable factor.

more, the fractions 6 and 7 seemed bound to the presence of this coagulogen but were not evident in each animal plasma. However, it must be noted that all these anticoagulant-dependent proteins, except 5, occurred in small amounts with faint precipitate lines, which were sometimes difficult to detect.

Analogous results were obtained in *Jasus lalendei.* Both crossed and line immunoelectrophoreses showed that some serine protease inhibitors acted upon the blood to prevent its gelation in some cases, since the clottable factor was evident in plasma with a trypsin inhibitor. Moreover, two fractions cross-reacting more or

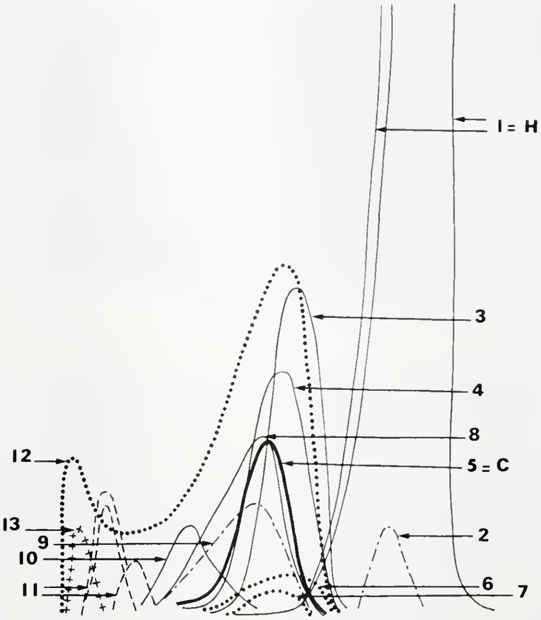


FIGURE 3. Schematic representation of crossed immunoelectrophoresis of the hemolymph from *Homarus vulgaris*. H shows the hemocyanin and C represents the clottable factor.

less completely with the fibrinogen of the NEM sample were observed in the PMSF H_2O hemolymph (Fig. 5).

DISCUSSION

In lobsters and spiny lobsters, hemolymph coagulation includes morphological changes in amoebocytes, followed by a release reaction, a cellular aggregation, and a beginning of retraction. This cellular transformation is rapidly hidden by the gelling of the whole plasma, which forms an "unretracted" clot. With inhibitors which delay whole-plasma clotting, but do not inhibit amoebocyte aggregation, complete retraction of the cellular clump is observed, recalling the thrombosthenin effect of vertebrate platelets. When plasma clotting is prevented, the "fibrinogen-like factor" always appears as a polymer scale in acrylamide gradient gels (minimum four bands), but as a single precipitate line in crossed immunoelectrophoresis. This electrophoretic heterogeneity is in agreement with the ultracentrifugation analysis of Duchateau and Florkin (1954) and Stewart *et al.* (1966), who have found both 21 S and 17–18 S components in the blood of *Homarus*. Fuller and Doolittle (1971a) have reported on the presence of 14.5 S and 19.4 S fibrinogen in *Panulirus interruptus*. Moreover, the latter authors have shown through disc electrophoresis that fibrinogen A is itself heterogeneous. With our specimens, DC situates these polymers from about 420,000 daltons (above 16 S hemocyanin) up to the top of the acrylamide gel. Fuller and Doolittle (1971a, b) and Doolittle and Fuller (1972), indicate the same weight for the "monomer" (fibrinogen B) of *Panulirus*. However, our "dimer" is not a multiple of 420,000, since the weight increase is of about 200,000 daltons between each of the four lighter bands. This could

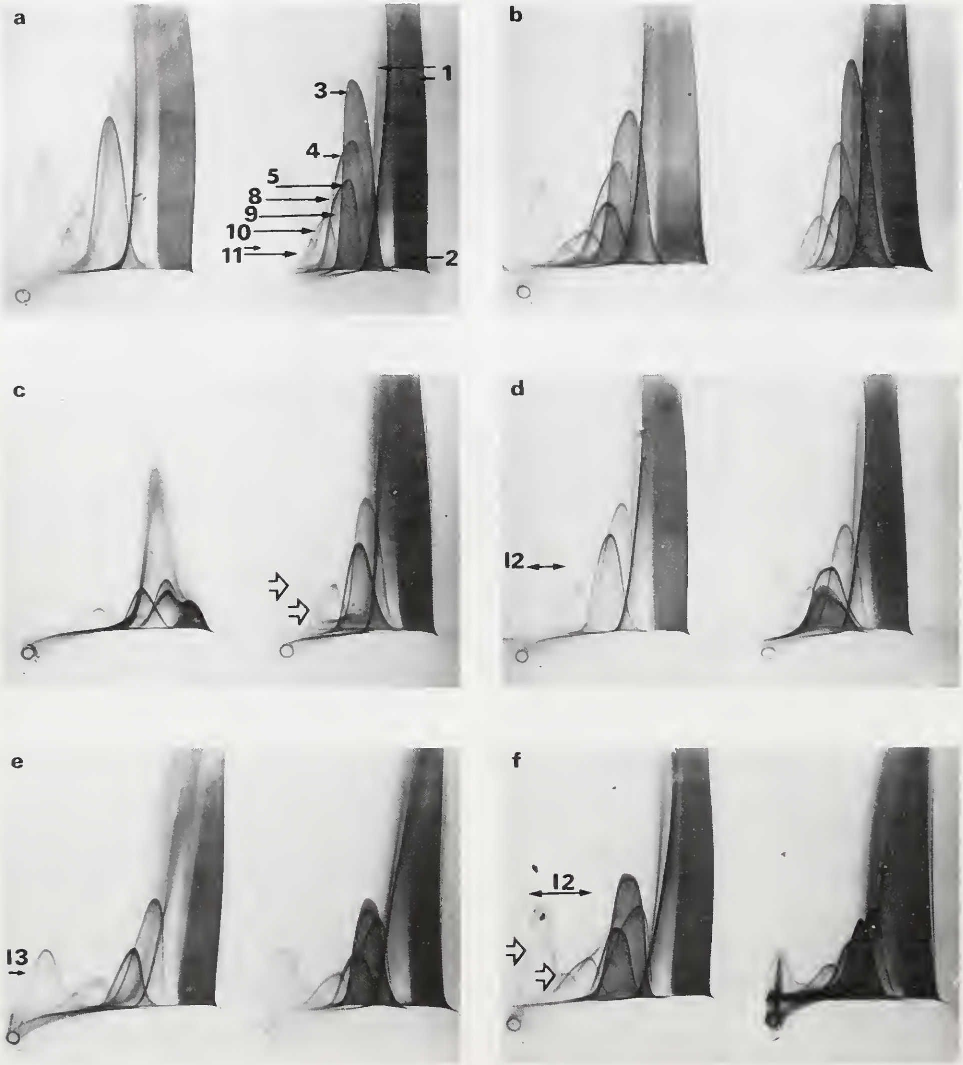


FIGURE 4. Crossed immunoelectrophoresis of hemolymphs from *Homarus vulgaris*. (a) Samples of serum and plasma with sodium citrate NaCl. (b) Plasmas with sodium citrate H_2O and NEM sodium citrate. (c) Samples with PMSF H_2O (dissociated after dialysis against a stripping buffer) and PMSF NaCl after centrifugation. (d) Plasmas with propanolol without and after centrifugation. (e) Plasmas with DC without and after centrifugation. (f) Samples with GME without and after centrifugation. The white arrow shows cross-reacting fractions of group 11. Each sample of serum and plasma contains 100 μg of proteins—An agarose gel containing 4 $\mu l/cm^2$ of lobster antiplasma serum is used.

perhaps better explain the shift from 18 S to 21 S evidenced through ultracentrifugation in *Homarus*. The pore gradient gel gives a true estimation of the molecular weights, since Fuller *et al.* (1972) have shown by electron microscopy that the molecule of "fibrinogen" of *Panulirus interruptus*, at least for the monomer, is spheroid.

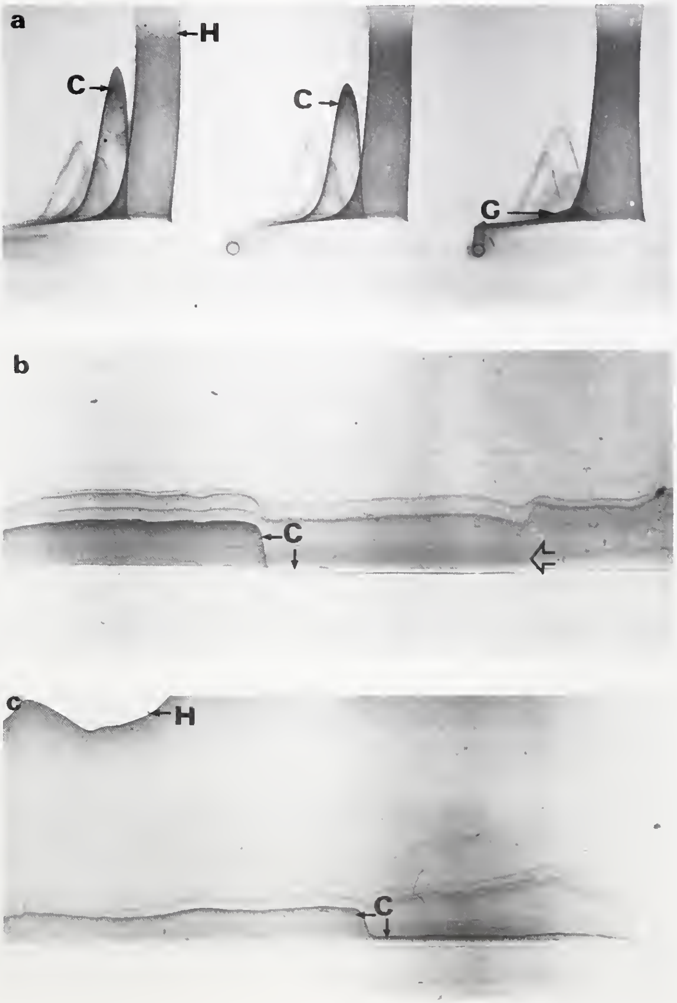


FIGURE 5. Immunoelectrophoreses of hemolymphs from *Jasus lalendei*. An agarose gel containing 15 $\mu l/cm^2$ of spiny lobster antiplasma serum is used. (a) Crossed immunoelectrophoresis: from left to right plasmas with concentrated GME, diluted GME, and trypsin inhibitor; each antigen contains 300 μg of plasma proteins. (b) and (c) Line immunoelectrophoreses: (b) From left to right plasmas with NEM, PMSF H_2O, and PMSF propanol. (c) From left to right samples of plasmas with GME and trypsin inhibitor. Antigens contain 600 μg of proteins in each line. H shows the hemocyanin and C represents the clottable factor. The arrow shows a fraction cross-reacting with the fibrinogen-like factor.

In supernatants separated from intact hemocytes (with saline solutions of NEM sodium citrate, PMSF, GME, or DC), one additional fraction appears when experiments are carried out for 48 h with a buffer at pH 9.5. In *Homarus* this fraction has approximately 240,000 daltons. Since the fluorescence of DC conjugates then is partly located at this place, it is possible that this component represents either the true monomer of "fibrinogen" or a subunit of soluble fibrin. This result is in agreement with the earliest reports of Fuller and Doolittle (1971), who have shown that the clottable factor is built up from two subunits of about 200,000 daltons.

However this band is not found in plasmas of *Jasus lalendei* and *Panulirus regius* (except in NEM solutions) although the polymer scale shows the same pattern as in *Homarus*.

With amines the clotting time of plasmas of *Homarus vulgaris* and spiny lobsters is considerably prolonged by the inhibition of fibrin crosslinkings, as reported by Lorand *et al.* (1963, 1964, 1965) in *Homarus americanus*. When low doses of GME or DC are used, the higher number of bands visible in the polymer scale leads us to think that the coagulation is initiated.

Indeed, this scale might not be the visualization of a biological phenomenon, but only the result of the experimental conditions since hemocyanin from *Homarus*, observed mainly as 24 S in ultracentrifugation, appears chiefly as 16 S and 5 S in our electrophoreses. In spiny lobsters, one supplementary band occurs in some samples (Table III), especially when 16 S is dissociated. It is probably a stable dimer (7 S) of subunits of hemocyanin. Dissociations of the native 24 S and 16 S hemocyanins during electrophoresis could be not only related to pH changes, but also connected with the ionic strength of the buffer and with the control of its temperature (Busselen, 1970). Thus, the ratio between the low ionic strength of the buffer and higher strength of the internal medium of sea animals could explain why we never observe 24 S in electrophoreses of the *Homarus* hemolymph. However, some other marine Crustacea (*Maia squinado, Cancer pagurus*) show 24 S hemocyanin in gradient gel electrophoresis. With a buffer system able to dissociate oligomeric proteins (hemocyanin and perhaps the fibrinogen-like factor) more or less completely during electrophoresis, the pattern of whole hemolymphs can vary according to migration time. Freezing promotes polymerization of coagulogen, sometimes to the point of complete clotting, for instance, with samples in DC after several freeze-thaw cycles. Since "fibrinogen" shows several peaks in ultracentrifugation, the polymer scale does not seem due to artifacts. These polymers are converted into insoluble fibrin by a transglutaminase enzyme, as in vertebrate clotting. However, no particular fraction related to cellular transamidase has been identified, perhaps due to the small amount of this protein. In crossed immunoelectrophoresis, changes in some fractions have been noted according to the sample studied, but their variations do not seem related to this enzyme. Thus, the fractions of group 11 observed only in female lobsters and spiny lobsters very likely represent vitellogenesis-related proteins, and fraction 2 could be dependent on the molting stage.

The macruran coagulation process seems more complex than previously claimed, since different inhibitors act upon the several steps of this plasma clotting. With serine protease inhibitors, plasma clotting is either totally absent (sometimes using DIFP, the trypsin inhibitor or, as in Joubert, 1954, the phosphate buffer) or delayed and abnormal. The soft gel, when formed, is easily dissociated mechanically, probably due to some clotting defects. However, results vary according to the animals investigated, perhaps in relation to their molting stages. Indeed, with the trypsin inhibitor, when plasma clotting is prevented in *Jasus lalendei*, the bands of the polymer scale are present, as with other anticoagulants, but are finer and slightly shifted towards higher molecular weights (Fig. 2b). A small proteolysis could occur on the clottable factor, as it does on the cellular coagulogen of *Tachypleus* (Nakamura *et al.*, 1976) and *Limulus* (Tai *et al.*, 1977), which was at first found not to be enzymatically cleaved (Solum, 1970). Thus, in presence of the soy bean trypsin inhibitor, these polymers might perhaps represent aggregates of "fibrinogen," even though the other polymer scales could be the visualization of uncrosslinked polymers of fibrin. It is exciting to think that the fibrinogen might be a short-lived transitory step, and that the circulating state could be soluble fibrin complexes. The protamine sulfate gelation test, which precipitates the soluble complexes of fibrin from vertebrates (Gurewich and Hutchinson, 1971), does not give similar results in these Decapoda. Nevertheless, whether the circulating state is fibrin or fibrinogen, this clottable factor should be called coagulin and its precursor coagulogen, as defined by Needham (1970), and more precisely plasmatic coagulogen and coagulin in order to differentiate them from cellular coagulogen and coagulin of *Limulus.*

These inhibitors also act at the cellular level, since they more or less prevent the clumping of hemocytes. It seems that some factors of coagulation are located within the amoebocytes and perhaps within the granules, since propanolol retards the plasma gelling. Analogous facts are reported on *Limulus* (Mürer *et al.*, 1975). Cell lysates from lobsters form a gel, which behaves as do coagulated cell lysates of *Limulus* (Solum, 1973). Cell lysates of *Homarus* seem to contain a coagulogen and an enzymatic system able to convert this coagulogen into a gel, which includes the aggregate cellular mass. This cellular clot, which is considerably delayed by inhibitors affecting serine hydroxyl and sulfhydryl groups, has been found in all Decapoda investigated (*Maia squinado, Cancer pagurus, Carcinus maenas, Xantho, Macropipus puber, Astacus leptodactylus, Homarus vulgaris, Nephrops norvegicus* and different species of spiny lobsters), whether or not they exhibit plasma clotting. This reaction is more or less strong and rapid according to the species studied (important in *Maia, Cancer*, and *Limulus polyphemus*) and is related to the molt stage and to the physiological state of the subjects (unpublished results).

In conclusion, a cellular coagulation analogous to that of the *Limulus* has been shown in the Decapoda studied. Although thrombin (a serine protease) does not promote plasma clotting of lobster hemolymphs, our results suggest that a thrombin-like enzyme, in addition to transglutaminase, may be involved in this process. Macruran plasmatic clotting seems to depend on a multienzymatic system. These enzymes could perhaps act in cascade-like sequences, as in some vertebrate coagulation mechanisms.

LITERATURE CITED

Bruner-Lorand, J., T. Urayama, and L. Lorand. 1966. Transglutaminase as a blood clotting enzyme. *Biochem. Biophys. Res. Comm.* **23:** 828–834.

Busselen, P. 1970. The electrophoretic heterogeneity of *Carcinus maenas* hemocyanin. *Arch. Biochem. Biophys.* **137:** 415–420.

Doolittle, R. F., and G. M. Fuller. 1972. Sodium dodecyl sulfate polyacrylamide gel electrophoresis studies on lobster fibrinogen and fibrin. *Biochem. Biophys. Acta* **263:** 805–809.

Doolittle, R. F., and L. Lorand. 1962. Inhibitors of lobster blood clotting. *Biol. Bull.* **123:** 481.

Duchateau, G., and M. Florkin. 1954. La coagulation du sang des Arthropodes. IV. Sur le fibrinogène et sur la "coaguline" musculaire du Homard. *Bull. Soc. Chim. Biol.* **36:** 295–305.

Durliat, M. 1974. Etude de la coagulation de l'hémolymphe chez les Crustacés Décapodes. Analyse électrophorétique des protéines du sérum et du plasma d'écrevisse (*Astacus leptodactylus*). *C. R. Acad. Sci. Paris, Ser. D* **278:** 473–476.

Durliat, M., and R. Vranckx. 1976. Coagulation in the crayfish, *Astacus leptodactylus*: attempts to identify a fibrinogen-like factor in the hemolymph. *Biol. Bull.* **151:** 467–477.

Fuller, G. M., and R. F. Doolittle. 1971a. Studies of invertebrate fibrinogen. I. Purification and characterization of fibrinogen from spiny lobster. *Biochemistry* **10:** 1305–1310.

Fuller, G. M., and R. F. Doolittle. 1971b. Studies of invertebrate fibrinogen. II. Transformation of lobster fibrinogen into fibrin. Biochemistry **10:** 1311–1315.

Fuller, G. M., J. D. McLean, and R. F. Doolittle. 1972. Electron microscopy of lobster fibrinogen and fibrin. Pp. 200–202 in Acton *et al.*, Eds., *Invertebrate Immune Defense Mechanisms*, M. S. S. Information Corporation.

Glavind, J. 1948. Studies on the coagulation of crustacean blood. Pp. 1–137 in A. Busck, Ed., *Nyt Nordisk Forlag.* Kjøbenhavn, Copenhagen, Denmark.

Gregoire, C. 1971. Hemolymph coagulation in Arthropods. Pp. 145–189 in M. Florkin and B. T. Scheer, Eds., *Chemical Zoology*, 6, Part B, Academic Press, New York and London.

Gurewich, V., and E. Hutchinson. 1971. Detection of intravascular coagulation by a serial dilution protamine sulfate test. *Ann. Internat. Med.* **75:** 895–902.

Hawkey, C. M. 1970. Fibrinolysis in animals. Pp. 133–150 in R. G. Mac Farlane, Ed. *Mechanisms in man and other animals. Symp. Zool. Soc. Lond.* **27.** Academic Press, New York and London.

Joubert, F. J. 1954. Haemocyanin of the crawfish (*Jasus lalandii*). *Biochim. Biophys. Acta* **14:** 127–135.

Krøll, J. 1973. Crossed-line immunoelectrophoresis. In quantitative immunoelectrophoresis. *Scand. J. Immunol.,* **2**(suppl. 1): 79–81.

Levin, J. 1967. Blood coagulation and endotoxin in invertebrates. *Fed. Proc.* **26:** 1707–1712.

Levin, J., and F. B. Bang. 1968. Clottable protein in *Limulus*: its localization and the kinetics of its coagulation by endotoxin. *Thromb. Diath. Heamorrh.* **19:** 186–197.

Lorand, L. 1972. Fibrinoligase: the fibrin-stabilizing factor system of blood plasma. *Ann. N. Y. Acad. Sci.* **202:** 6–30.

Lorand, L., R. F. Doolittle, K. Konishi, and S. K. Riggs. 1963. A new class of blood coagulation inhibitors. *Arch. Biochem. Biophys.* **102:** 171.

Lorand, L., A. Jacobsen, and A. Schuel. 1964. Inhibitors of fibrin polymerization. *Biol. Bull.* **127:** 379.

Lorand, L., T. R. E. Pilkington, and J. Bruner-Lorand. 1965. A search for new inhibitors of fibrin crosslinking. *Biol. Bull.* **129:** 413.

Mosesson, M. W., C. Wolfenstein-Todel, J. Levin, and O. Bertrand. 1979. Characterization of amoebocyte coagulogen from the horseshoe crab (*Limulus polyphemus*). *Thromb. Res.* **14:** 765–779.

Mürer, E. H., J. Levin, and R. Holme. 1975. Isolation and studies of the granules of the amebocytes of *Limulus polyphemus*, the horseshoe crab. *J. Cell. Physiol.* **86:** 533–542.

Myhrman, R., and J. Bruner-Lorand. 1970. Lobster muscle transpeptidase. *Methods Enzymol.* **19:** 765.

Nakamura, S., T. Takagi, S. Iwanaga, M. Niwa, and K. Takahashi. 1976. A clottable protein (coagulogen) of horseshoe crab hemocytes. Structural change of its polypeptide chain during gel formation. *J. Biochem.* **80:** 649–652.

Needham, A. E. 1970. Haemostatic mechanisms in the invertebrata. Pp. 19–44 in R. G. Mac Farlane, Ed. *Mechanisms in man and other animals. Symp. Zool. Soc. Lond.* **27:** Academic Press, New York and London.

Shishikura, F., J. Chiba, and K. Sekiguchi. 1977. Two types of hemocytes in localization of clottable protein in Japanese horseshoe crab, *Tachypleus tridentatus. J. Exp. Zool.* **201:** 303–308.

Shishikura, F., and K. Sekiguchi. 1978. Comparative study on horseshoe crab coagulogens. *J. Exp. Zool.* **206:** 241–246.

Solum, N. O. 1970. Some characteristics of the clottable protein of *Limulus polyphemus* blood cells. *Thromb. Diath. Haemorrh.* **23:** 170–181.

Solum, N. O. 1973. The coagulogen of *Limulus polyphemus* hemocytes. A comparison of the clotted and non-clotted forms of the molecule. *Throm. Res.* **2:** 55–70.

Stewart, J. E., J. R. Dingle, and P. H. Odense. 1966. Constituents of the hemolymph of the lobster *Homarus americanus* Milne Edwards. *Can. J. Biochem.* **44:** 1447–1459.

Sullivan, J. D., and S. W. Watson. 1975. Purification and properties of the clotting enzyme from *Limulus* lysate. *Biochem. Biophys. Res. Comm.* **66:** 848–855.

Tai, J. Y., and T. Y. Liu. 1977. Studies on *Limulus* amoebocyte lysate. I. Isolation of pro-clotting enzyme. *J. Biol. Chem.* **252:** 2178–2181.

Tai, J. Y., R. C. Seid, R. D. Huhn, and T. Y. Liu. 1977. Studies on *Limulus* amoebocyte lysate—II—Purification of the coagulogen and the mechanism of clotting. *J. Biol. Chem.* **252:** 4773–4776.

Tait, J., and D. J. Gunn. 1918. The blood of *Astacus fluviatilis*: a study in crustacean blood, with special reference to coagulation and phagocytosis. *Quart. J. Exp. Physiol.* **12:** 35–80.

Tyler, A., and B. T. Scheer. 1945. Natural heteroagglutinins in the serum of the spiny lobster, *Panulirus interruptus.* II. Chemical and antigenic relation to blood proteins. *Biol. Bull.* **89:** 193–200.

Vranckx, R., and M. Durliat. 1978. Comparison of the gradient of setal development of uropods and of scaphognathites in *Astacus leptodactylus. Biol. Bull.* **155:** 627–639.

Young, N. S., J. Levin, and R. A. Prendergast. 1972. An invertebrate coagulation system activated by endotoxin. Evidence for enzymatic mediation. *J. Clin. Invest.* **51:** 1790–1797.

Reference: *Biol. Bull.*, **160:** 69–79. (February, 1981)

FIBER COMPOSITION AND INNERVATION PATTERNS OF THE LIMB CLOSER MUSCLE IN THE LOBSTER *HOMARUS AMERICANUS*

C. K. GOVIND, T. W. BUDD, AND H. L. ATWOOD

Scarborough College and Department of Zoology, University of Toronto, West Hill, Ontario, Canada M1C 1A4; and Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543

ABSTRACT

The closer muscle in the first walking leg of the lobster, *Homarus americanus*, is composed of mostly (78%) tonic fibers with low levels of myofibrillar adenosine triphosphatase (ATPase) activity. The remainder are phasic fibers, which have high ATPase activity and are restricted to a dorsal bundle. Based on histochemical demonstration of reduced nicotinamide dinucleotide (NADH) diaphorase, phasic fibers have a lower oxidative capacity than tonic fibers. The tonic fibers have mitochondria criss-crossing the entire cross-sectional profile or restricted to the periphery.

Fiber types based on myosin ATPase activity correlated closely with those based on resting sarcomere and A-band lengths on the dorsal surface of the closer muscle. Thus phasic fibers with high ATPase activity had short (<6 μm) sarcomeres and tonic fibers with low ATPase activity had long (>6 μm) sarcomeres. Innervation patterns of the dorsal fibers revealed all possible combinations between the two types of muscle fibers (phasic and tonic) and the two types of axons (fast and slow) except phasic fibers innervated only by slow axons. The distribution of fast and slow axons therefore serves to broaden the contractile performance of the closer muscle.

INTRODUCTION

The organization of the motor system in the thoracic appendages of decapod crustaceans is relatively simple (Wiersma, 1961; Atwood, 1973, 1976). Each of the three distal segments has two major antagonistic muscles innervated by relatively few (2–5) motoneurons. These few motoneurons bring about the full range of movements of the limbs. It is therefore not surprising to find them differentiated into fast and slow types. The neuromuscular synapses arising from each type of motoneuron vary in their transmitter release and facilitation effects on individual muscle fibers. Furthermore, muscle fibers are specialized into three broad types: phasic, tonic, and intermediate. Thus differentiation of motoneurons, neuromuscular synapses, and muscle fibers provides the basis for a wide range of contractile behaviour.

At one extreme of this range is the rapid twitch contraction produced by fast axons innervating phasic fibers, as in deep abdominal extensor and flexor muscles in crayfish (Kennedy and Takeda, 1965a, b). At the other extreme is the slow tonic contraction brought about by slow axons innervating tonic fibers, as in the crayfish superficial abdominal muscles. The closer muscle in the first two pairs of walking

---

Received 12 August 1980, accepted 8 November 1980.

Abbreviations: FCE, fast closer excitor motoneuron; SCE, slow closer excitor motoneuron; ATPase, adenosine triphosphatase; NADH, nicotinamide adenine dinucleotide; ejp, excitatory junctional potential.

legs of the lobster (*Homarus americanus*) produces each type of contraction upon stimulation of its fast closer excitor (FCE) and slow closer excitor (SCE) motoneurons respectively (Wiersma, 1955). However, we do not know whether the different contractions can be accounted for by phasic and tonic fibers. Nor do we know the extent to which the distribution of innervation by each of the axons regulates contractile behaviour. The present study addresses these questions.

Materials and Methods

Lobsters (*Homarus americanus*) weighing 300–500 g were used throughout this study. The larger animals were purchased locally in Toronto and kept in artificial sea water at 10–11°C. The smaller animals were captured in the waters around Woods Hole and kept in running sea water at ambient temperatures. The closer muscle in the first and second walking legs were examined with the techniques described below.

Histochemistry

We followed the methods described by Ogonowski and Lang (1979) for detection of myofibrillar adenosine triphosphatase (ATPase) and reduced nicotinamide adenine dinucleotide (NADH) diaphorase in lobster muscle. Briefly, an intact propus of a walking leg was frozen and serially cross-sectioned (15 μm thick). The sections were placed on glass cover slips and stained by modifications of the methods of Padykula and Herman (1955) for myofibrillar ATPase and of Nachlas *et al.* (1958) for NADH diaphorase. The stained sections were mounted on glass slides for subsequent examination and photography. The muscles in propuses of the first walking legs from three separate lobsters were assayed histochemically.

The average number of fibers in the closer muscle was estimated by counting the number of fast and slow fibers (based on intensity of staining for ATPase) in three cross-sections from each of the proximal, central, and distal regions of the muscle. The three regions were far enough apart so that fibers did not overlap.

Light microscopy

Muscle fiber types were also determined by measuring the average resting length of the sarcomeres and A-bands in the myofibrils by the following technique. The dorsal surface of a closer muscle was exposed by removing the overlying opener muscle and the muscle was held at rest length, by appropriate manipulation of its dactyl, while being fixed in alcoholic Bouins fluid for 24 h. Muscle fibers were teased into fine strands in 70% alcohol on glass slides and viewed at 600 magnification under a light microscope. Five consecutive sarcomeres and three A-bands were measured with an ocular micrometer to obtain an average value for each fiber (Govind *et al.*, 1977). A total of 75 fibers were sampled in two separate closer muscles.

Electrophysiology

The innervation of muscle fibers by FCE and SCE axons was determined using conventional electrophysiological techniques. Recording from fibers of the closer muscle was done in lobster physiological saline of the following composition: NaCl, 472 m*M*; KCl, 10 m*M*; $MgCl \cdot 6H_2O$, 5 m*M*; CaCl, 16 m*M*; glucose, 11 m*M*; Tris maleate, 10 m*M*; pH 7.4. The saline was continuously perfused over the preparation at a rate of about 200 ml/h and kept at 12–15°C (Meiss and Govind, 1979).

The dorsal aspect of the closer muscle was exposed (Fig. 1) by removing the antagonistic opener muscle and the connective tissue separating the two muscles. This also exposes the major branches of the closer nerve on the muscle surface. The main closer nerve was stimulated with a pair of fine platinum wire electrodes, near the point where it divides in the carpus. The resulting action potentials were monitored with an extracellular suction electrode on a primary branch of the closer nerve. At the same time, excitatory junctional potentials (ejp's) were recorded from individual muscle fibers by intracellular glass microelectrodes filled with 2 *M* KAc solution and having resistances of 5–15 MΩ. Brief square wave pulses of 0.1–0.15 msec were applied to the stimulating electrodes. Under these conditions the preparations were viable for at least 4–8 h. A total of 15 preparations were made from 12 separate lobsters.

RESULTS

Fiber composition based on enzyme histochemistry

Two distinct types of fibers were defined on the basis of different staining intensities for myofibrillar ATPase in cross-sections of the closer muscle (Fig. 2A–D). The intensely staining fibers demonstrated high activity for this enzyme, which is characteristic of phasic muscle (Hajek *et al.*, 1973; Lehman and Szent-Györgi, 1975). Conversely, fibers staining less intensely denoted low activity for myofibrillar ATPase characteristic of tonic muscle. Thus the closer muscle in the first walking leg of lobster is composed of phasic and tonic fibers. The proportions of phasic and tonic fibers were estimated to be 22% and 78% respectively.

Phasic and tonic fibers also have a regional distribution in the closer muscle. The phasic fibers originate as a dorsal bundle on either side of the tendon in the distal part of the pinnate muscle (Fig. 2C, D) and insert on the exoskeleton in the proximal part (Fig. 2A, B).

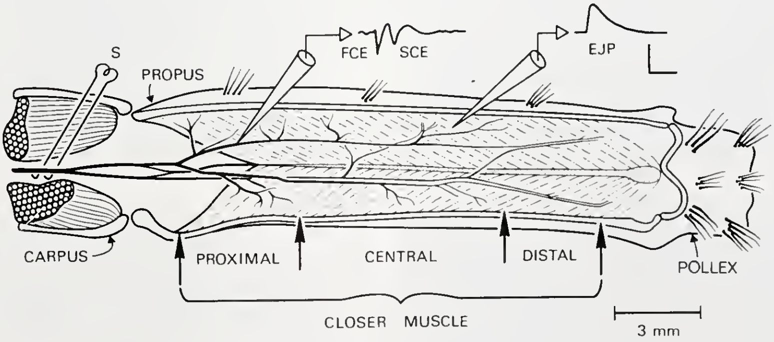
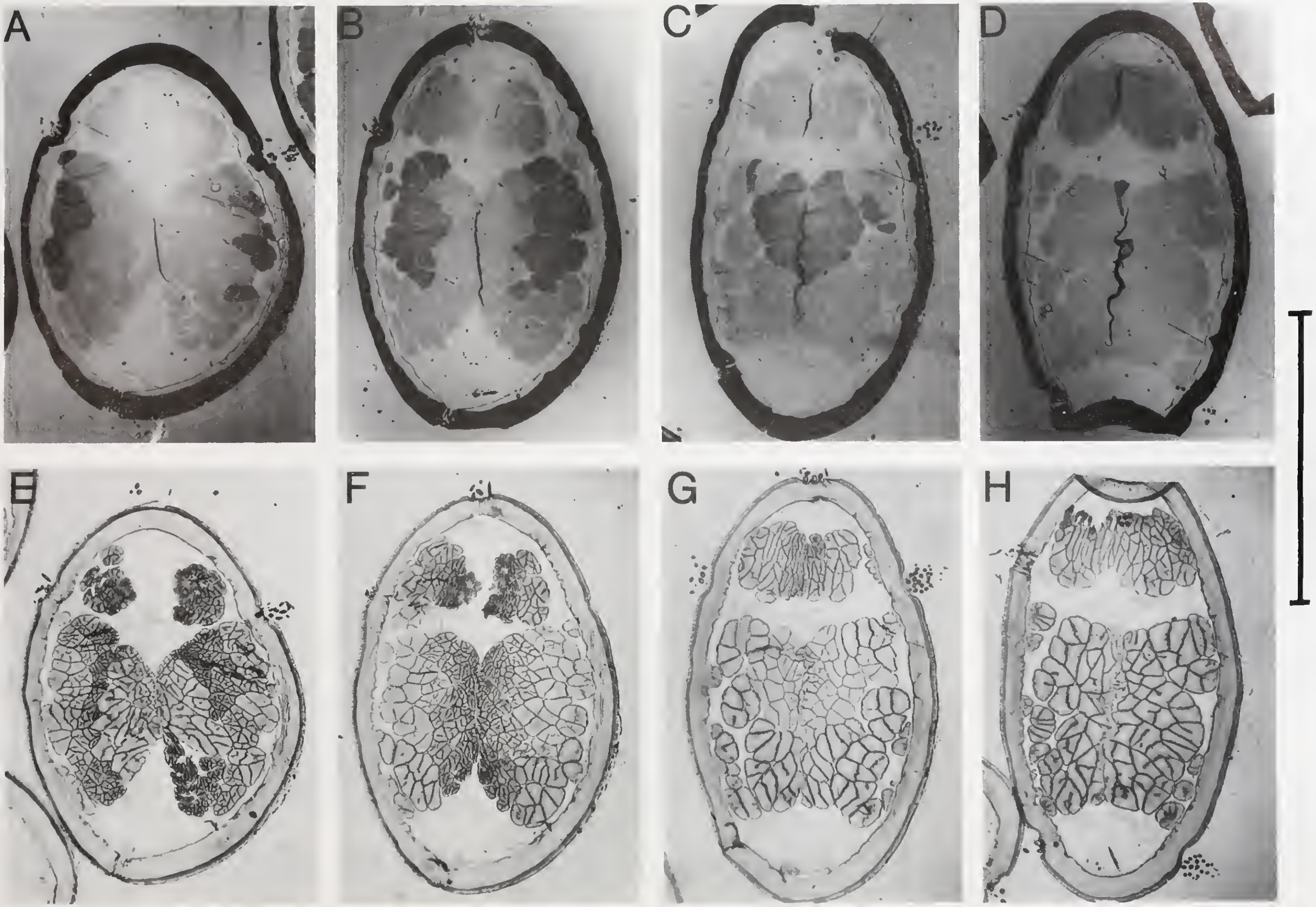


FIGURE 1. Dorsal aspect of the closer muscle in the first walking leg of lobster, *Homarus*, showing subdivision of the fibers into those at the ends of the muscle, *i.e.* proximal and distal, and those between, *i.e.* central. The branching pattern of the closer nerve as revealed by methylene blue staining shows the main nerve in the carpus dividing into two branches which ramify over the dorsal surface of the muscle and also send branches to the ventral areas. Stimulation (S) of the main nerve in the carpus evokes in one of the primary branches two extracellularly recorded action potentials, the faster conducting of which was identified as the FCE axon and the slower conducting as the SCE axon. Simultaneous intracellular recording from one of the central fibers gave an EJP in response to FCE stimulation but not to SCE stimulation. Calibration bar; vertical 5 mV; horizontal 30 msec.


A
B
C
D
E
F
G
H

Alternate cross-sections of the propodite were stained for NADH diaphorase to measure oxidative capacity (Fig. 2E–H). The diformazan granules formed denoted the density and location of mitochondria in the muscle fibers. In general, phasic fibers had a lower oxidative capacity than tonic fibers. Thus in the proximal and central parts of the closer muscle (Fig. 2E–G) fibers with the weakest staining reaction for NADH diaphorase have the same location as those staining intensely for ATPase (Fig. 2A–C). These are phasic fibers. Fibers staining more heavily for NADH diaphorase in the distal part (Fig. 2H) are the same ones that stain weakly for ATPase. These are tonic fibers. The tonic fibers also have more pronounced staining of their boundaries than the phasic fibers (Fig. 2G), denoting greater concentration of subsarcolemmal mitochondria. Thus in the closer muscle there appears to be good correlation between fiber type based on ATPase activity and the oxidative capacity of the fiber, *i.e.*, phasic fibers have a low oxidative capacity and tonic fibers a high oxidative capacity.

Among the tonic fibers the staining reaction for NADH diaphorase shows one of two patterns (Fig. 3). In some fibers the staining is restricted principally to the periphery of the fiber while in others it also criss-crosses the fiber. The proximal region of the closer muscle (Fig. 2E, F) has both types of tonic fibers whereas the distal area (Fig. 2G, H) has fibers with the mitochondria distributed along their boundaries. Phasic fibers also have their mitochondria restricted largely to the periphery (Fig. 3), where they appear in lower concentration than those in tonic fibers.

Since cross-sections of the entire propodite were taken, the antagonistic opener muscle was also treated histochemically, along with the closer muscle (Fig. 2). Fibers of this muscle stained lightly for myofibrillar ATPase, indicating that low activity is characteristic of tonic muscle (Fig. 2A–D). Alternate sections stained for NADH diaphorase (Fig. 2E–H) indicated relatively high oxidative capacity with some regional variation: Fibers in the proximal part stained more intensely (Fig. 2E, F) than those in the distal part (Fig. 2G, H).

In summary, based on myofibrillar ATPase activity, the closer muscle in the lobster walking leg appears to be composed of a minority of phasic fibers, restricted to a dorsal bundle, and a majority of tonic fibers. The phasic fibers have a lower oxidative capacity than the tonic fibers, which themselves have variable oxidative capacities.

Fiber composition based on sarcomere length

Fibers on the dorsal surface of the closer muscle were characterized by their resting sarcomere lengths, which reliably indicate contractile speed: Phasic fibers have relatively short sarcomeres (2–4 μm) and tonic fibers longer sarcomeres (>6 μm) (Atwood, 1973, 1976). The resting sarcomere length can vary considerably depending on the stretch or contraction of the fiber when fixed. Consequently, the length of the A-band was measured as a precaution. Though changes in the length

FIGURE 2. Representative cross sections of the propus from the first walking leg of lobster, *Homarus*, stained for myofibrillar ATPase (A–D) and NADH diaphorase (E–H). The opener muscle is located dorsally and is small compared to the closer muscle occupying most of the propus. Adjacent sections were stained for ATPase and NADH diaphorase and these are shown from the proximal (A, E), central (B, C, F, G), and distal (D, H,) regions. Scale mark, 5 mm.

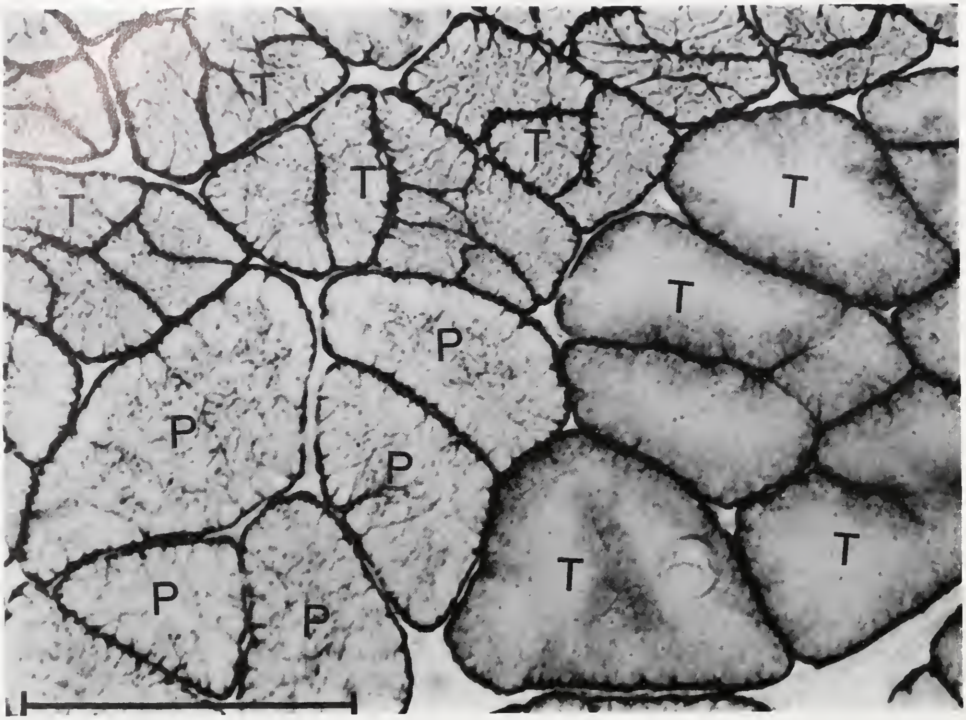


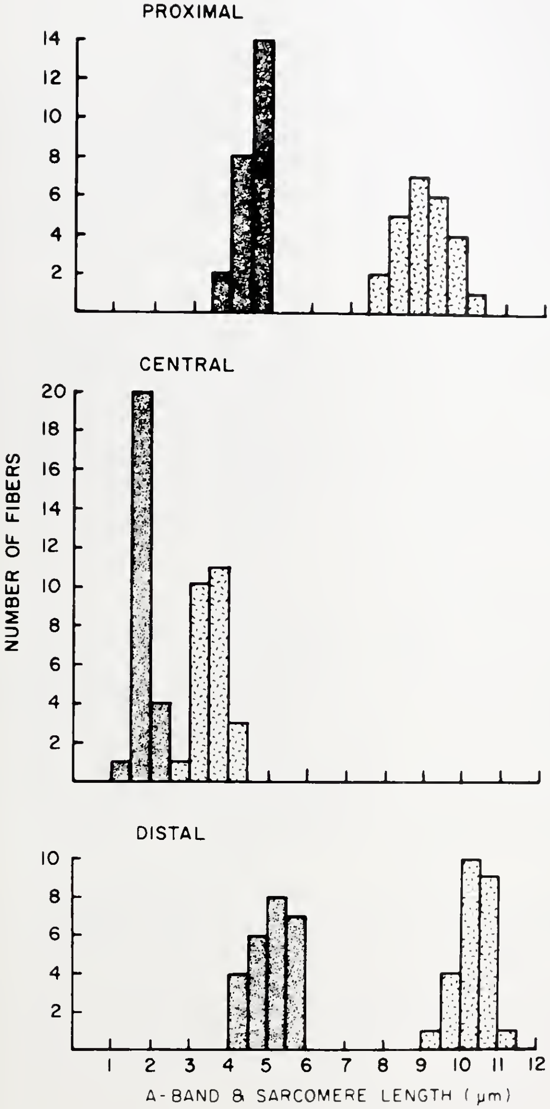
FIGURE 3. Enlarged view of part of Figure 2F showing cross-section of closer muscle fibers stained for NADH diaphorase. Staining is restricted to the circumference in all phasic (P) and some tonic (T) fibers; in other tonic (T) fibers the staining subdivides the fiber extensively, denoting a more widespread distribution of mitochondria. Scale mark, 0.5 mm.

of the A-band have been reported during contraction (Dewey *et al.*, 1977) they are so minute as to escape detection by conventional measuring techniques.

Whereas the determination of fiber types based on enzyme histochemistry dealt with the entire closer muscle, that based on sarcomere length was restricted to the dorsal surface (Fig. 1). For determining sarcomere length, fibers were taken from the two ends of the muscle, *i.e.*, proximal and distal areas, and from the center, thus avoiding overlap in sampling among the three regions. A total of 25 fibers were examined from each area. Two distinct types of fibers were found, and these occurred regionally (Fig. 4). Fibers with short sarcomeres and A-bands characteristic of phasic type occurred in the central region, while those with long sarcomeres and A-bands characteristic of tonic type were at the two ends of the muscle (Fig. 4).

Innervation by FCE and SCE axons

Innervation received by muscle fibers from the two excitor axons, FCE and SCE, on the dorsal surface of the closer muscle was determined by electrophysiological techniques. Since the FCE axon has a higher conduction velocity than the SCE axon (Wiersma, 1955; Hoyle and Wiersma, 1958; Govind and Lang, 1974), its action potential preceded that of the SCE axon in extracellular recordings from the closer nerve (Fig. 1). Recordings of muscle excitatory junctional potentials



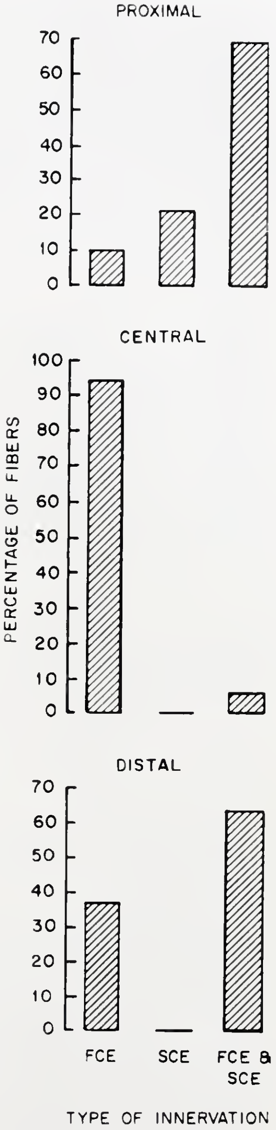


FIGURE 4. (Left) Distribution of fiber types based on A-band (darker stipple) and sarcomere length (light stipple) of proximal, central, and distal fibers from the dorsal surface of the closer muscle in first walking leg of lobster, *Homarus*.

FIGURE 5. (Right) Histogram of percent innervation by FCE, SCE, and both FCE and SCE axons in proximal, central, and distal fibers from the dorsal surface of the closer muscle in the first two pairs of walking legs of lobster, *Homarus*.

(ejp's) corresponding to each action potential confirmed that they were excitor axons. Selective stimulation of FCE and SCE axons was done in one of two ways. Either the two axons were physically separated in the carpus or each was recruited at a lower voltage by appropriate placement of the stimulating electrode. In order to determine whether a muscle fiber received endings from an axon, the axon was stimulated repetitively at frequencies ranging from 1–50 Hz with unpatterned pulses and from 10–30 Hz with twin pulses. The patterned stimulation was performed to facilitate ejp's so that they could be readily detected.

The sampling procedure followed for the sarcomere length measurement was adopted for the innervation studies, *i.e.*, only fibers from the center and the two ends of the dorsal surface were examined. Figure 5 shows the results from 121 fibers: 38 proximal, 53 central, and 30 distal fibers. The most striking observation is that almost all (94%) of the central fibers received only the FCE axon, while the majority of distal and proximal fibers received both FCE and SCE axons. Overall, the FCE axon innervated 54% of the dorsal fibers, the SCE axon 6%, and both axons 40%.

Since the main purpose of the study was to correlate occurrence of innervation with muscle fiber types, no attempt was made to examine the neuromuscular synapses qualitatively. However, maximum ejp's evoked by each axon at 1 Hz stimulation decreased from proximal to distal fibers. Thus for the FCE axon the largest ejp was 15 mV in a proximal fiber, 6.2 mV in a central fiber, and 1 mV in a distal fiber. The results suggest that synapses on the proximal fibers have a higher quantal release of transmitter at low frequencies than their counterparts on the distal fibers. This phenomenon needs to be studied further.

DISCUSSION

Correlation between ATPase activity and oxidative capacity

The present histochemical analysis of the closer muscle in the first walking leg of lobster revealed that myofibrillar ATPase activity is correlated with oxidative capacity. Thus phasic fibers with high ATPase activity have low oxidative capacity, while tonic fibers with low ATPase activity have high oxidative capacity. Such correlations are typical for other crustacean muscles (Ogonowski and Lang, 1979; Ogonowski *et al.*, 1980), insect muscle (Stokes *et al.*, 1979), and vertebrate muscle (Close, 1973).

However, within each fiber type, *i.e.*, phasic and tonic, there is further differentiation with regards to oxidative capacity. Among the fast-contracting fibers this is manifested by fast-fatiguing and fatigue-resistant types in mammals (Burke *et al.*, 1971) and insects (Stokes *et al.*, 1979). Fast-contracting fibers may be similarly differentiated in crustaceans. Thus the phasic deep-abdominal extensor and flexor (Ogonowski and Lang, 1979) and the cutter closer muscles in the lobster are of the fast-fatiguing variety, whereas the phasic maxilliped muscle in blue crab is a fatigue-resistant muscle (Silverman and Charlton, 1980).

Among slow-contracting fibers, which are characteristically fatigue-resistant in vertebrates, a fast-fatiguing variety has been found in the mesocoxal muscles of cockroaches (Stokes *et al.*, 1979). Our studies on lobster muscle have revealed at least two types of tonic fibers based on enzyme histochemistry. The most convincing demonstration of such differentiation is found among muscles composed entirely of tonic fibers, such as the claw opener muscle and the closer muscle of the crusher claw (K. S. Kent, Boston Univ., unpublished). In these muscles a few fibers have much lower myofibrillar ATPase activity and much higher oxidative capacity than the other tonic fibers. The present study on the closer muscle in the first walking leg of lobster revealed at least two types of tonic fibers, based on their distribution of mitochondria.

In addition, differences in oxidative capacity were initially seen in the lobster claw closer muscle (Lang *et al.*, 1980) and correlated with innervation by the FCE and SCE axons. Thus fibers receiving only SCE endings had high oxidative capacity, those receiving only the FCE had low oxidative capacity, and fibers receiving both axons had intermediate capacity. However, differences in oxidative capacity among fibers of the opener muscle (K. S. Kent, unpublished; and present study), which has a single tonic motoneuron, argues against the view that the type of motoneuron directly regulates oxidative capacity of the fiber it innervates.

Correlation between ATPase activity and sarcomere length

It is worthwhile to compare the classification of crustacean muscle fiber types based on enzyme histochemistry (Ogonowski and Lang, 1979; Silverman and Charlton, 1980), and by their resting sarcomere length (Atwood, 1973, 1976). In the present study both techniques yielded similar results in the classification of fiber types on the dorsal surface of the limb closer muscle. Thus fibers in the central area, which stained intensely for myofibrillar ATPase (an indication of phasic fibers), also had short sarcomere (2–4 μm) and A-band lengths. Conversely, the distal and proximal fibers, which stained lightly for myofibrillar ATPase had long sarcomere (8–11 μm) and A-band lengths. A similar correlation between fiber types obtained by these two techniques has also been found in the lobster claw closer (Lang *et al.*, 1980; Ogonowski *et al.*, 1980) and abdominal extensor and flexor (Ogonowski and Lang, 1979) muscles, and in a blue crab mouthpart muscle (Silverman and Charlton, 1980). Clearly, both techniques may be used to determine muscle fiber types in crustaceans; and they complement each other nicely, since each measures a separate contractile property.

Correlation between fiber type and innervation

The closer muscle in the walking leg of lobster is composed of phasic and tonic fibers (present study) and is supplied with an FCE and SCE motoneuron. Each axon may therefore innervate the two fiber types separately or together, and in this way potentially extend the range of contractile behaviour of a single muscle. The manner and extent to which this occurs were examined for the dorsal fibers of the closer muscle. Based on enzyme histochemistry and sarcomere length, fibers of the central area are phasic, while those of the proximal and distal regions are tonic. In the central region almost all (94%) were innervated by the FCE axon exclusively, providing a neuronal basis for fast contraction. These same fibers demonstrated high myofibrillar ATPase activity, providing an enzymatic basis for fast contractions (Lehman and Szent-Gyorgi, 1975). Furthermore, the short sarcomeres characteristic of these central fibers provide a structural basis for fast contractions, as the speed of shortening is directly related to the number of sarcomeres in series per unit length of the fiber (Huxley and Niedergerke, 1955; Jahromi and Atwood, 1969). Thus, in terms of their innervation and fiber characteristics, the central fibers would constitute a phasic unit within the closer muscle. Whether the location of this unit within the closer muscle is mechanically especially favorable for producing fast contractions remains to be determined.

In other areas of the closer muscle the two types of motoneurons (FCE and SCE) innervate the two types of muscle fibers (phasic and tonic) in all possible combinations except the one where phasic fibers are innervated by SCE only. In functional terms, therefore, the closer muscle has five different contractile units, all serving to enhance its behavioural repertoire.

In the present study phasic fibers did not receive the SCE axon exclusively. Nor was this combination found in the homologous closer muscle in the cutter claw of lobster (Lang *et al.*, 1980). Since this is the only combination of neuron and muscle fiber type not found in the closer muscle, it may be important in revealing how motoneurons select their target muscle fibers or how muscle fibers regulate their innervation.

ACKNOWLEDGMENTS

We thank Dr. Harold Silverman for criticism of the manuscript, and Joanne Pearce and Lena Hill for technical assistance. Supported by grants from NSERCC and Muscular Dystrophy Association of Canada to C.K.G. and H.L.A. and from NIH (NINCDS) to Dr. A. G. Humes (Director of Boston University Marine Program). T.W.B. received a fellowship from the Government of Ontario.

LITERATURE CITED

ATWOOD, H. L. 1973. An attempt to account for the diversity of Crustacean muscle. *Am. Zool.* **13:** 357–378.

ATWOOD, H. L. 1976. Organization and synaptic physiology of crustacean neuromuscular systems. *Prog. Neurobiol.* **7:** 291–391.

BURKE, R. E., D. N. LEVINE, F. E. ZOJAC, P. TSAIRIS, AND W. K. ENGEL. 1971. Mammalian motor units: Physiological histochemical correlations in three fiber types in cat gastrocnemius. *Science* **174:** 709–712.

CLOSE, R. E. 1973. Dynamic properties of mammalian skeletal muscle. *Physiol. Rev.* **52:** 129–197.

DEWEY, M. M., B. WALCOTT, D. E. COLEFLESH, H. TERRY, AND R. J. C. LEVINE. 1977. Changes in thick filament length in *Limulus* striated muscle. *J. Cell Biol.* **75:** 366–380.

GOVIND, C. K., AND F. LANG. 1974. Neuromuscular analysis of closing in the dimorphic claws of the lobster, *Homarus americanus. J. Exp. Zool.* **190:** 281–288.

GOVIND, C. K., J. SHE, AND F. LANG. 1977. Lengthening of lobster muscle fibres by two age-dependent mechanisms. *Experientia.* **32:** 1170–1171.

HAJEK, I., N. CHARI, A. BASS, AND E. GUTMANN. 1973. Differences in contractile and some biochemical properties between fast and slow abdominal muscles of the crayfish (*Astacus leptodactylus*). *Physiol. Bohemslov.* **22:** 603–612.

HOYLE, G., AND C. A. G. WIERSMA. 1958. Excitation at neuromuscular junctions in Crustacea. *J. Physiol. (Lond.)* **143:** 403–425.

HUXLEY, A. F., AND R. NIEDERGERKE. 1955. Structural changes in muscle during contraction. Interference microscopy of living muscle fibers. *Nature* **173:** 971–973.

JAHROMI, S. S., AND H. L. ATWOOD. 1969. Correlation of structure, speed of contraction and total tension in fast and slow abdominal muscles of the lobster (*Homarus americanus*). *J. Exp. Zool.* **171:** 25–38.

KENNEDY, D., AND K. TAKEDA. 1965a. Reflex control of abdominal flexor muscles in the crayfish. I. The twitch system. *J. Exp. Biol.* **43:** 211–227.

KENNEDY, D., AND K. TAKEDA. 1965b. Reflex control of abdominal flexor muscles in the crayfish. II. The tonic system. *J. Exp. Biol.* **43:** 229–246.

LANG, F., M. M. OGONOWSKI, W. J. COSTELLO, R. HILL, B. ROEHRIG, K. KENT, AND J. SELLERS. 1980. Neurotrophic influence on lobster skeletal muscle. *Science* **207:** 325–327.

LEHMAN, W., AND A. G. SZENT-GYORGI. 1975. Regulation of muscular contraction: distribution of actin control and myosin control in the animal kingdom. *J. Gen. Physiol.* **66:** 1–30.

MEISS, D. E., AND C. K. GOVIND. 1979. Regional differentiation of neuromuscular synapses in a lobster receptor muscle. *J. Exp. Biol.* **79:** 99–114.

NACHLAS, M. M., D. G. WALKER, AND A. M. SELIGMAN. 1958. A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase. *J. Biophys. Biochem. Cytol.* **4:** 29–38.

OGONOWSKI, M. M., AND F. LANG. 1979. Histochemical evidence for enzyme differences in crustacean fast and slow muscle. *J. Exp. Zool.* **207:** 143–151.

OGONOWSKI, M. M., F. LANG, AND C. K. GOVIND. 1980. Histochemistry of lobster claw closer muscles during development. *J. Exp. Zool.* **213:** 359–367.

PADYKULA, H. A., AND E. HERMAN. 1955. Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques. *J. Histochem. Cytochem.* **3:** 161–169.

SILVERMAN, H., AND M. P. CHARLTON. 1980. A fast-oxidative crustacean muscle: histochemical comparison with other crustacean muscle. *J. Exp. Zool.* **211:** 267–273.

STOKES, D. R., A. J. VITALE, AND C. R. MORGAN. 1979. Enzyme histochemistry of the mesocoxal muscles of *Periplaneta americana. Cell Tiss. Res.* **198:** 175–189.

WIERSMA, C. A. G. 1955. An analysis of the functional differences between the contraction of the adductor muscles in the thoracic legs of the lobster, *Homarus vulgaris* L. *Arch. Neerl. Zool.* **11:** 1–13.

WIERSMA, C. A. G. 1961. The neuromuscular system. Pp. 191–240 in T. H. Waterman, Ed., *The Physiology of Crustacea*, Vol. II. Academic Press, New York.

Reference: *Biol. Bull.*, **160:** 80–88. (February, 1981)

THE REPRODUCTIVE CYCLE OF EARLY SETTING *CRASSOSTREA VIRGINICA* (GMELIN) IN THE NORTHERN GULF OF MEXICO, AND ITS IMPLICATIONS FOR POPULATION RECRUITMENT

PAUL F. HAYES AND R. WINSTON MENZEL

Department of Oceanography, Florida State University, Tallahassee, Florida 32306

Abstract

Oysters which set early in the spawning season reach sexual maturity and spawn before the end of their first year. The early sexual development in the southern latitudes is the result of both an extended growing season and an increased instantaneous rate of growth. Widespread spawning of oysters older than one year occurs twice during the reproductive season, with a period of renewed development in between. Spawning by young-of-the-year oysters is of minimal importance to population recruitment during the year they set.

Introduction

Numerous studies have been conducted to determine the annual reproductive cycle of *Crassostrea virginica* at various locations throughout its range. The majority of these studies were conducted along the northern Atlantic coast and clearly demonstrate that in the northern latitudes only one generation of oysters is produced annually. In contrast, several authors (Burkenroad, 1931; Coe, 1938; Menzel, 1951; Butler, 1954) have suggested the possibility of multiple generations within each year in this species' southern range.

The present paper reports on field studies conducted to describe the reproductive cycle and breeding patterns of *C. virginica* in southern latitudes. Of particular interest was when spawning occurred in oysters which had set early in the breeding season.

Materials and Methods

Sampling sites

In April 1978 two sampling stations were established in the northeastern Gulf of Mexico. One site, Turkey Point, was near the mouth of a small stream and the other, Alligator Harbor, in a neutral estuary. Temperature and salinity were measured at each station before sampling.

Sampling the established oyster populations

Each station had a fairly discrete, well established oyster population along the vertical walls of private boat docks. These oysters were sampled to monitor the gonad condition of the established populations and were the progenitors for establishing the young-of-the-year populations.

At first, we sampled the established populations at each station weekly. Later, when the young population was established, sampling was approximately biweekly.

Received 30 January 1980, accepted 21 October 1980.

On each sampling date groups of attached oysters were obtained from a randomly selected area.

Samples were returned to the laboratory on ice and stored at 4°C until processed.

Sampling the young-of-the-year populations

As cultch material holders, we built wooden racks, each holding a maximum of 30 asbestos shingles (hereafter panels; each 30 cm × 60 cm) 5 cm apart. Grooves cut in the racks allowed removal and addition of panels. To minimize predation, the racks were held off the bottom.

A full rack was positioned near the established oyster population at each station on 20 May 1978. This was the first time the water temperature exceeded 25°C at both sites and corresponds to the expected time of the spring mass spawning (Hopkins, 1931; Ingle, 1951; Menzel, 1955). After 1 week under water, each panel had received an abundant fall of spat. This served as the young-of-the-year populations for the remainder of the study. Alternate panels were removed from these racks on 26 September to provide unrestricted space for further growth.

Sampling young populations began after the spat had reached about 20 mm in shell height because sizes smaller than 20 mm were impossible to process reliably. We know of no practical method to determine exactly which spat settled first, so we used the traditional method of selecting the largest specimens as the oldest (those in the initial set) (Ingle and Dawson, 1952; Menzel, 1955).

After the spat were large enough for sampling, collections were approximately biweekly, by removing the panel furthest from the dock. The panel, with oysters attached, was brought to the laboratory on ice and stored at 4°C until processed.

Sample processing

Within 72 h of sampling, we arbitrarily selected from the established populations 25 oysters larger than the largest specimen from the young population, to insure that only oysters from previous years' setting would be sampled. Each of these oysters was separated, measured (sensu Galtsoff, 1964), shucked, weighed, and fixed whole in Bouin's fixative.

The largest 25 specimens on each panel were individually labeled and maintained separately throughout the laboratory procedure outlined above.

After at least 72 h in Bouin's fixative, a 5–10 mm section was removed from mid-way between the anterior end of the oyster and the anterior side of the adductor muscle perpendicular to the anterior-posterior body axis. Standard histological preparations were made, following Humason (1967).

Slide analysis

We used the quantitative method for evaluating the gonadal condition of oysters described by Tinsman *et al.* (1976), with slight modifications. After determining the percent gonad and gonad density for each specimen, a follicle index was calculated by multiplying these measurements together. This index measures the area devoted to germinal tissue in each oyster cross section, which in turn measures the oyster's gonadal development (Tinsman *et al.*, 1976).

Each specimen also was examined with a light microscope (250×) to assign it to a particular stage of development by the more traditional procedure (Loosanoff and Engle, 1942).

Determination of setting patterns

A second cultch-holding rack was constructed for each station and positioned adjacent to the rack for holding the developing spat. Each week a clean panel was inserted into the rack to provide fresh setting material, and the panel exposed during the previous week was removed and examined to determine the density of spat attachment. Random coordinates were used to place a 7 cm × 7 cm transparent plastic grid on the panel. A stereoscope (10×) with an extendible arm was used to determine the number of spat in four areas on each side of the panel.

Statistical analysis

A distribution-free multiple comparison test based on Kruskal-Wallis rank sums was used to determine significant differences between the mean follicle indexes within each population (Hollander and Wolfe, 1973).

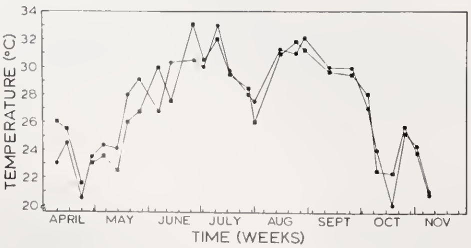
RESULTS

Temperature

Figure 1 shows water temperature patterns at the Turkey Point and Alligator Harbor sites. The seasonal temperature changes at both stations were similar, with the largest difference on any sampling date about 3°C. Between 20 May and 5 October the temperature remained above 25°C. The temperature drop of about 6°C at both stations in July is attributed to increased storms and rainfall. A low of 20°C and a high of 33°C were recorded during the experiment.

Salinity

Due to the nature of the locations, salinity fluctuated more at Turkey Point than at Alligator Harbor. On every sampling date except one (29 July), the salinity at Turkey Point was lower than that at Alligator Harbor. Both stations experienced slightly higher salinity from September to November. The salinity varied from 15.0 to 32.3‰ at Turkey Point and from 27.0 to 33.7‰ at Alligator Harbor.



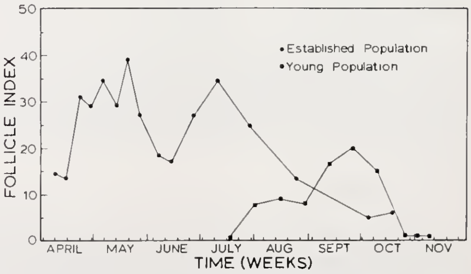


FIGURE 1. (Left.) Surface water temperature at each station for all sampling dates. Measurements were made with a stick thermometer read to the nearest 0.1°C. Circles = Turkey Point, squares = Alligator Harbor.

FIGURE 2. (Right.) Gonadal condition of the established and young populations at Turkey Point. Each point is the median follicle index of the 25 specimens for that date (except the mid-July young population point, which is the median of 15 specimens). The median gave the best representation of the central tendency of the data due to skewed distributions.

Growth

Growth of the young populations was initially faster at Alligator Harbor than at Turkey Point. However, little growth was observed at the former from mid-August through September, resulting in a smaller average size at Alligator Harbor by the end of the experiment (9 November). Turkey Point had an initially slower, but overall a more steady rate of growth.

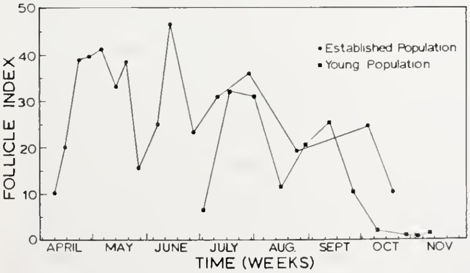
If setting is assumed to have occurred on the first day the cultch material was set out (20 May), an average growth rate of 0.79 mm/day at Alligator Harbor and 0.45 mm/day at Turkey Point was recorded for the 25 fastest growing individuals from setting until the initial sampling (3 and 18 July, respectively). By the end of the experiment, the average size of the 25 largest individuals from the young population at Alligator Harbor was 65.9 mm, a daily growth rate of 0.38 mm. At Turkey Point the average shell length of the 25 largest individuals at the end of the study was 84.1 mm, a growth of 0.47 mm/day.

The fastest growing oyster early in the study was one in Alligator Harbor, which reached 43 mm in the 44 days from when setting could have first occurred, a growth rate of 0.98 mm/day. Toward the end of the study one Turkey Point specimen had reached 105.0 mm in 166 days. This represents a growth rate of 0.63 mm/day.

Follicle index

The follicle index data, which measure gonadal development, are presented in graphic form in Figure 2 (Turkey Point) and Figure 3 (Alligator Harbor). The established populations at both stations followed similar trends over the study period, rising during April and early May, declining in late May and early June, recovering again until mid- to late July, and finally declining until the project ended in November. The large, statistically significant peak on 14 June at Alligator Harbor appears to be anomalous: It does not follow the trend at Turkey Point, nor does the developmental stage data support it as an indication of the population trend.

Statistical analyses of the follicle index data for the established populations support the stated interpretations except for one point: The drop in the index at



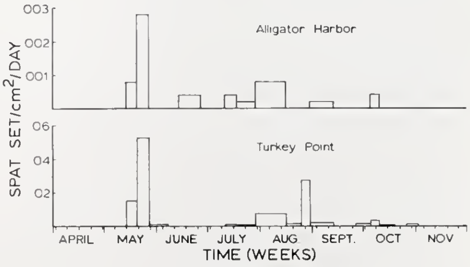


FIGURE 3. (Left.) Gonadal condition of the established and young populations at Alligator Harbor. Each point is the median follicle index of the 25 specimens for that date. The median gave the best representation of the central tendency of the data due to a skewed distribution.

FIGURE 4. (Right.) Oyster setting patterns at both stations. Each bar represents the average number of oyster spat counted in eight randomly chosen 49 cm^2 areas on the panel exposed during the period covered by the width of the bar. Note scale differences.

Turkey Point from 20 May to 14 June is not significant, whereas the corresponding decrease at Alligator Harbor is significant.

The follicle index for the young-of-the-year populations also changed similarly at the two sampling sites. Both sites' indexes increased sharply in July and early August. This rise was followed by a small decrease at Turkey Point and a large one at Alligator Harbor. Both indexes rose again until mid- to late September, when they peaked, and then declined steadily until the project ended. Statistical analyses confirmed the significance of the trends stated above, except for the slight decrease at Turkey Point in late August. The decline at Alligator Harbor during this period was significant.

Developmental stage

The percent of individuals in five general stages of development, determined by traditional techniques, yield some additional information to support the more quantitative results. Data from the established population show that gonads were active throughout the duration of the study. At least some of these oysters spawned from the end of April to the end of October.

The developmental-stage data for the two young populations show active gonads in Alligator Harbor from early July to early November and at Turkey Point from early August to early November. Most of the spawning appears to have occurred during September and October, although some did take place as early as August or even July at Alligator Harbor, and as late as November.

Setting patterns

Figure 4 summarizes the setting data from both stations. The overall setting trends at both stations are fairly similar. A large setting peak occurred during the period 20 May–27 May at both stations. Setting continued at a much lower level until late July and mid-August when a smaller setting peak occurred. After August, some light setting was recorded at both stations until the project ended in early November.

Discussion

Examination of gonads from over 1300 *C. virginica* specimens enabled the first quantitative determination of this species' reproductive cycle in the Gulf of Mexico.

Observations of the gonadal condition of naturally established oyster populations demonstrate great dependence on water temperature. Gonadal development in the area studied begins before the temperature reaches 20°C in April, probably sometime in March or late February (Gennette and Morey, 1971; Ingle, 1951; Menzel and Hopkins, 1953). Although some spawning was observed before the water reached 25°C, most took place afterwards. This was clearly indicated by the decrease of the follicle index for the established populations during late May. Although the statistical basis for this conclusion is obscured by some asynchronization at Turkey Point, the trend is significant at Alligator Harbor. Gonadal condition differed somewhat on every sampling date, particularly in the established populations, demonstrating that synchronization is never 100%.

This study also demonstrated that established oysters in the Gulf of Mexico begin new development of gametes after the initial spring spawning, as previously suggested by Hopkins *et al.* (1953), and Gennette and Morey (1971). This continues as the dominant trend during the summer, as long as the temperature remains high.

Temperature fluctuations of 5–10°C also can induce spawning (Loosanoff and Davis, 1963). During late July to early August, a temperature drop large enough to induce spawning was recorded. This temperature change appears to have begun the steady late-summer decline of the follicle index, indicative of increased spawning. In years when this temperature change does not occur, widespread spawning may not begin again until the fall temperature decline in September.

This study is the first to describe the reproductive cycle of young-of-the-year *C. virginica.* The results clearly support the hypothesis that two generations are produced in this area each year. Increases in the follicle index as well as the presence of more advanced developmental stages indicate that early setting oysters ripen at both stations during the summer. This process appears to have been slightly enhanced at Alligator Harbor, where growth was also faster at first. Active gonads were found there 2 weeks earlier than at Turkey Point.

Some of these young oysters spawned during mid-summer, apparently in response to temperature fluctuation. This suggests that some specimens were mature enough for natural spawning to occur only 12 weeks after attachment. When the temperature rose again, it appears that gonadal development resumed, as it had in the established oysters after the spring spawning. The September fall temperature decline initiated large-scale spawning in the young oysters. The reproductive cycle for these young oysters includes active gonads from late June or early July until late October. These oysters spawn to some degree throughout this period, mostly in late September and early October.

Orton (1920) and Giese (1959) have stated that the occurrence of more than one generation each year in the warmer areas of a species' distribution could be due to accelerated development and/or an extended period when conditions are suitable for gonadal development. Which permits or causes multiple generations to occur in *C. virginica* in the warmer areas of its distribution is not known.

Numerous reports on the growth rate of *C. virginica* in various areas of its distribution demonstrate that oysters grow faster in the warmer areas (Gunter, 1951; Ingle, 1950; Ingle and Dawson, 1952; Loosanoff, 1965a; Maurer and Aprill, 1973; Shaw and Merrill, 1966). Pruder *et al.* (1976), presenting theoretical growth expectations for a mariculture operation based on the fastest growth data available in the north, calculated a maximum growth rate equal to about half that recorded in our study. Dame (1972) also showed that in South Carolina the instantaneous growth rate for all sizes examined is fastest during summer. Data in the literature support the idea of faster instantaneous growth rates in the southern latitudes.

Numerous studies on various aspects of oyster physiology present conflicting results describing the optimal environment for oysters. Some studies indicate the temperature of maximal feeding, water pumping, or heart rate to be about 25°C, while others show peaks at approximately 30–35°C (Collier, 1954; Federighi, 1929; Galtsoff, 1927, 1964; Loosanoff, 1958, 1965b; Nelson, 1935). Although the basal metabolic demand of many organisms increases with increasing temperature (Kinne, 1963), Vernberg and Vernberg (1970) and Dame (1972) indicated that the Q_{10} value for growth of *C. virginica* is much lower during the warmer period than during the winter months. Low Q_{10} values at intermediate and higher temperatures imply energy utilization without excessive waste (Dame, 1972). Dame (1972) also reported higher assimilation rates for South Carolina oysters during summer.

The importance of a longer season of suitable growing conditions cannot be overlooked when examining the causes of rapid development of southern oysters. However, it appears that these locations also present a more favorable environment for increased rates of instantaneous growth and assimilation than do northern locations. These faster rates are of paramount importance for promoting the observed rapid sexual development.

Although many factors affect oyster setting, the amount of spawning activity ultimately determines setting potential and the maximum possible recruitment to an oyster population. Comparing the spawning and setting data of the young and established populations at each station gives some idea of relative contributions to recruitment.

Most of the setting recorded in this study occurred during the spring, in late May. This peak can be attributed solely to oysters attached in previous years. Setting later in the season, however, may be due to additional spawning by the older oysters and/or spawning of the sexually developed young-of-the-year oysters. The results of this study demonstrate that not only are the older oysters solely responsible for early setting, but they contribute most of the gametes that result in later setting. Numerous studies of setting patterns in northern areas, where early sexual development does not occur, have shown a bimodal distribution of setting activity (Chestnut, 1948; Chestnut and Fahy, 1952; Hopkins, 1937; Loosanoff, 1966; McNulty, 1953; Shaw, 1967). These studies support the hypothesis that older oysters can spawn several times during the reproductive season or that some spawn early while others spawn later in the season (Galtsoff, 1964). The present study indicates significant spawning by the older oysters both in the spring and the fall, with continual light spawning throughout the summer. Also, the observation of renewed sexual development in older oysters during the summer provides these specimens with more potential for spawning in the fall than if renewed gametogenesis had not occurred.

Although the young oysters undoubtedly contribute to the mid-summer setting peak (as demonstrated by the presence of spawning individuals), no significant peak was observed when most of them were spawning in response to the fall temperature decrease. Thus, the young oysters, although spawning by the end of their first year, make little significant contribution to the overall population recruitment during that year. This is undoubtedly due to their relatively smaller size and fewer numbers compared to oysters from previous years.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Drs. W. H. Heard, M. J. Greenberg, and W. C. Burnett for reviewing the manuscript and for loaning us equipment. J. Mandrup-Poulsen and S. Glomb also deserve our thanks for criticizing various revisions of the manuscript. This work was supported in part by a Sigma Xi Grant-in-aid of Research and a grant from the Florida State University Eric Vicklund Fund, both to the senior author.

LITERATURE CITED

BURKENROAD, M. D. 1931. Sex in the Louisiana Oyster *Ostrea virginica*. *Science* **74**: 71–72.

BUTLER, P. A. 1954. Summary of our knowledge of the oyster in the Gulf of Mexico. *U. S. Fish. Wildl. Serv. Bull.* **89**: 479–489.

CHESTNUT, A. F. 1948. Late summer and early fall spawning of oysters and its relation to sets of commercial value. *Nat. Shellf. Assoc. Conv. Addresses* **39**: 57–60.

CHESTNUT, A. F., AND W. E. FAHY. 1952. Studies on the setting intensity of oysters in Bogue Sound, North Carolina. *Proc. Nat. Shellf. Assoc.* **43**: 79–89.

COE, W. R. 1938. Primary sexual phases in the oviparous oyster (*Ostrea virginica*). *Biol. Bull.* **74:** 64–75.

COLLIER, A. 1954. A study of the response of oysters to temperature, and some long range ecological interpretations. *Proc. Nat. Shellf. Assoc.* **44:** 13–38.

DAME, R. F. 1972. The ecological energies of growth, respiration and assimilation in the intertidal American oyster *Crassostrea virginica. Mar. Biol.* **17:** 243–250.

FEDERIGHI, H. 1929. Temperature characteristics for the frequency of heartbeat in *Ostrea virginica* (Gmelin). *J. Exp. Zool.* **54:** 89–94.

GALTSOFF, P. S. 1927. The effect of temperature on the mechanical activity of the gills of the oyster (*Ostrea virginica* Gmelin). *J. Gen. Physiol.* **11:** 415–431.

GALTSOFF, P. S. 1964. The American oyster, *Crassostrea virginica* Gmelin. *U. S. Fish. and Wildl. Serv. Bull.* **64:** 1–480.

GENNETTE, A. F., AND S. W. MOREY. 1971. The effects of heated water on the gonadal development of the oyster *Crassostrea virginica* (G.). Pp. 98–104 in J. A. Quick, Jr., Ed., *A preliminary investigation: the effect of elevated temperature on the American oyster C. virginica (Gmelin). Professional Papers Series, No. 15.* Fla. Dept. Nat. Resources Mar. Res. Lab., St. Petersberg, Fla.

GIESE, A. C. 1959. Comparative physiology: annual reproductive cycles of marine invertebrates. Pp. 547–576 in V. E. Hall, Ed., *Annual Review of Physiology, Vol. 21.* Annual Reviews, Palo Alto.

GUNTER, G. 1951. The West Indian tree oyster on the Louisiana coast, and notes on growth of the three gulf coast oysters. *Science* **113:** 516–517.

HOLLANDER, M. AND D. A. WOLFE. 1973. *Nonparametric statistical methods.* John Wiley & Sons, New York. 503 pp.

HOPKINS, A. E. 1931. Factors influencing the spawning and setting of oysters in Galveston Bay, Texas. *Bull. Bur. Fish.* **47:** 55–58.

HOPKINS, A. E. 1937. Experimental observations on spawning, larval development, and setting in the Olympia oyster *Ostrea lurida. Bull. Bur. Fish.* **48:** 439–503.

HOPKINS, S. H., J. G. MACKIN, AND R. W. MENZEL. 1953. The annual cycle of reproduction, growth, and fattening in Louisiana oysters. *Proc. Nat. Shellf. Assoc.* **44:** 39–50.

HUMASON, G. L. 1967. *Animal tissue techniques.* W. H. Freeman and Co., San Francisco. 569 pp.

INGLE, R. M. 1950. Summer growth in the American oyster in Florida waters. *Science* **112:** 338–339.

INGLE, R. M. 1951. Spawning and setting of oysters in relation to seasonal environmental changes. *Bull. Mar. Sci. Gulf Carib.* **1:** 111–135.

INGLE, R. M., AND C. E. DAWSON, JR. 1952. Growth of the American oyster, *Crassostrea virginica* (Gmelin), in Florida waters. *Bull. Mar. Sci. Gulf and Carib.* **2:** 393–404.

KINNE, O. 1963. The effects of temperature and salinity on marine and brackish water animals 1. Temperature. Pp. 301–340 in H. Barnes, Ed., *Oceanogr. Mar. Biol. Ann. Rev., Vol. 1.* Allen and Unwin, London.

LOOSANOFF, V. L. 1958. Some aspects of behavior of oysters at different temperatures. *Biol. Bull.* **114:** 57–70.

LOOSANOFF, V. L. 1965a. The American or eastern oyster. *Bur. Comm. Fish., Circular 205*, 36 pp.

LOOSANOFF, V. L. 1965b. Gonad development and discharge of spawn in oysters of Long Island Sound. *Biol. Bull.* **129:** 546–561.

LOOSANOFF, V. L. 1966. Time and intensity of setting of the oyster, *Crassostrea virginica*, in Long Island Sound. *Biol. Bull.* **130:** 211–227.

LOOSANOFF, V. L., AND H. C. DAVIS. 1963. Rearing of bivalve mollusks. Pp. 1–136 in F. S. Russel, Ed., *Advances in Mar. Biol., Vol. 1.* Academic Press, New York.

LOOSANOFF, V. L., AND J. B. ENGLE. 1942. Accumulation and discharge of spawn by oysters living at different depths. *Biol. Bull.* **82:** 413–422.

MAURER, D., AND G. APRILL. 1973. Feasibility study of raft culture of oysters in the Delaware Bay area. *Report to Delaware River Basin Commission.* 180 pp.

MCNULTY, J. K. 1953. Seasonal and vertical patterns of oyster setting off Wadmalaw Island, S. C. *Contrib. from Bears Bluff Lab., No. 15*, 17 pp.

MENZEL, R. W. 1951. Early sexual development and growth of the American oyster in Louisiana waters. *Science* **113:** 719–721.

MENZEL, R. W. 1955. Some phases of the biology of *Ostrea equestris* Say and a comparison with *Crassostrea virginica* (Gmelin). *Instit. Mar. Sci.*, **4:** 69–153.

MENZEL, R. W., AND S. H. HOPKINS. 1953. Report on oyster experiments at Bay Sainte Elaine oil field. *Rept., Texas A & M Res. Found. Proj. 9.* 170 pp.

NELSON, T. C. 1935. Water filtration by the oyster and a new hormone effect thereon. *Anat. Rec.* **64:** 68.

ORTON, J. H. 1920. Sea-temperature, breeding and distribution in marine animals. *J. Mar. Biol. Assoc. U. K.* **12:** 339–366.

PRUDER, G. D., E. T. BOLTON, E. E. GREENHAUGH, AND R. E. BAGGALEY. 1976. Oyster growth and nutrient nitrogen cost in bivalve mulluscan mariculture. *Delaware Sea Grant Technical Report, Del-SG-11-76.* 20 pp.

SHAW, W. N. 1967. Seasonal fouling and oyster setting on asbestos plates in Brood Creek, Talbot County, Maryland, 1963–1965. *Ches. Sci.* **8:** 228–236.

SHAW, W. N., AND A. S. MERRILL. 1966. Setting and growth of the American oyster, *C. virginica*, on navigation buoys in the lower Chesapeake Bay. *Proc. Nat. Shellf. Assoc.* **56:** 67–72.

TINSMAN, J. C., S. G. TINSMAN, AND D. MAURER. 1976. Effects of a thermal effluent on the reproduction of the American oyster. Pp. 64–72 in G. W. Esch and R. W. McFarland, Eds., *Thermal ecology, Vol. 2. AEC Symposium Series (CONF-750425)*, Augusta, GA.

VERNBERG, F. J., AND W. B. VERNBERG. 1970. *The animal and the environment.* Holt, Reinhart & Winston, New York. 398 pp.

Reference: *Biol. Bull.*, **160:** 89–106. (February, 1981)

STUDIES ON THE ADULT REPRODUCTIVE DIAPAUSE OF THE MONARCH BUTTERFLY, *DANAUS PLEXIPPUS*[1]

WILLIAM S. HERMAN

Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108

ABSTRACT

Monarch butterflies obtained at monthly intervals during the annual adult cycle were held in summer conditions for 5, 10, and 20 days. Examination of these revealed in both sexes an adult reproductive diapause characterized by depressed growth of those reproductive organs sensitive to juvenile hormone. Diapause began for both sexes in late August or early September; female diapause ended in December while male diapause ended in November. Diapause intensity, maximal in September–October, appeared to be greatest in females. In summer conditions, reproductive tract growth in postdiapause females, but not males, was comparable to that observed in prediapause animals. Previously mated postdiapause, but not diapause, females showed a greater response to summer conditions than did virgins. Storing prediapause monarchs in winter conditions for up to 5 months did not induce diapause, indicating that diapause may be induced in a preadult stage. Diapause monarchs of both sexes responded to juvenile hormone with pronounced growth of the pertinent reproductive organs, suggesting that decreased production of juvenile hormone may be a characteristic of adult diapause. The possible advantages of diapause to the survival of the adult population are discussed.

INTRODUCTION

Most monarch butterflies (*Danaus plexippus plexippus*) in the northern United States are reproductively active from May until August, but the monarchs usually begin a period of reproductive inactivity before migrating southward during September and October (Urquhart, 1960; Brower, 1977). With apparently minor exceptions (Brower, 1961; Funk, 1968; Urquhart *et al.*, 1968), female reproductive tracts normally remain undeveloped until the following February or March, when the large overwintering colonies disperse and female reproductive tract development accelerates (Williams *et al.*, 1942; Urquhart, 1960). Similarly, monarch males have periods of depressed reproductive tract development during the overwintering period (Herman, 1975a). The existence of reproductive and non-reproductive adult stages in both sexes of this species strongly suggests an adult reproductive diapause during part of the adult cycle. Some direct evidence supports the existence of such a condition in both sexes (Herman, 1973, 1975a). However, previous studies have merely shown that the reproductive tracts of reproductively inactive overwintering monarchs develop in response to summer-like environments. They have not demonstrated a well-defined period of reduced reproductive tract development in favorable environmental conditions, *i.e.*, a reproductive diapause (Wigglesworth,

---

Received 7 April 1980, accepted 8 November 1980.

Abbreviations: LD25, regimen of 16 h light: 8 h dark at 25 C; SD10, regimen of 8 h light: 16 h dark at 10°C; JH, juvenile hormone; AJH, Ayerst mixture of juvenile hormone; MO, mature oocytes; OV, ovarian; CG, colleterial gland; AG, accessory glands; TG, tubular glands; ED, ejaculatory ducts.

[1] Supported by USPHS grant HD-07336 and funds from the University of Minnesota Graduate School.

1965), sandwiched between reproductively active stages of the adult life cycle. Thus, although a reproductive diapause at a specific stage of adult monarch life seems probable, its existence has not been conclusively demonstrated.

Against the above background, and as part of a continuing study of the neuroendocrinology of this lepidopteran, (Dallmann and Herman, 1978; Dores *et al.*, 1979; Johnson, 1979; Lessman, 1980), I have attempted to demonstrate and partially characterize the adult monarch reproductive diapause. Particular attention was given those organs of the male and female reproductive tracts known to be sensitive to juvenile hormone, *i.e.*, the female ovaries and colleterial glands, and the male accessory glands, tubular glands, and ejaculatory ducts (Pan and Wyatt, 1971; Barker and Herman, 1973; Herman, 1975a, b; Herman and Barker, 1977). These studies show an adult diapause at certain times in both sexes and indicate pronounced sexual differences during diapause. Furthermore, the data provide information dealing with the induction, duration, intensity, and endocrine regulation of this process.

MATERIALS AND METHODS

The monarchs used from June–August were obtained from larvae reared outdoors on milkweed (*Asclepias syriaca*) and allowed to pupate and emerge as adults in the laboratory. Those examined in September were captured in the Minneapolis, MN, metropolitan area. Butterflies studied from October–March were collected from a single large overwintering colony near Santa Cruz, CA, in at least 3 years. After capture the latter animals were immediately airmailed to MN, usually arriving without fatalities 2–4 days after capture. Most collections from the colony were made in the first week of each month. Major differences in body weight on arrival in laboratory, or in response to various experimental manipulations, were not observed in any single month from June–March. Animals were not examined in April–May, since in those months the butterflies have left the overwintering colonies, but none have emerged in MN. Except where otherwise noted, all monarchs were held individually in glassine envelopes and fed 30% honey daily.

Most investigations were conducted on animals stored in incubators at 25°C with a long day (LD) 16 h photophase provided by 40 W bulbs with average intensities of 435 lumens. Both male and female butterflies were held at this regimen (designated LD25, and considered comparable to summer conditions) for 10 days in most months of a 3-year period, and in at least 1 year for 5 and 20 days in most months. No data was obtained on March females held for 20 days at LD25, since in the year such studies were planned most females left the colony before the collection date. One set of experiments examined the effects of storage at 10°C with a short day 8 h photophase (SD10), considered comparable to winter conditions, while in another monarchs were held outdoors in the ambient September environment. The latter studies were performed over 3 years, principally during the first weeks of September. During that time, mean ambient temperature in the Minneapolis area normally declines from 19°C to 15°C, and the time from sunrise to sunset decreases from 13 hr:13 min to 12 hr:14 min.

Wet body and organ weights were measured to the nearest 0.1 and 0.01 mg, respectively, using a Sartorium Type 24-33 and an Ainsworth 24N balance, respectively. (Dissection and weighing procedures are described in Herman, 1975b.) Means of body or organ weights were rounded to the nearest mg or 0.1 mg, respectively. A direct relationship obtains between wet and dry weights in all pertinent monarch reproductive organs (Herman, 1975b; Johnson, 1979). This relationship was confirmed during this study. All mature oocytes (*i.e.*, those with ridged chorions) in both ovaries and in the remainder of the reproductive tract were counted in each female examined, and means rounded to the nearest single oocyte. The gross anatomy of male and female monarch reproductive tracts are described elsewhere (Urquhart, 1960; Herman, 1975b); they generally correspond to that found in other lepidopterans.

The Ayerst mixture (AY-22, 342-3) of juvenile hormone (JH) I mixed isomers (*i.e.*, AJH) was used in most studies in which exogenous hormone was applied. This material has high JH activity in both monarch sexes (Herman, 1975a, b; Herman and Barker, 1977; Lessman, 1979a). In some experiments all *trans, trans, cis* JH I, obtained from CalBiochem, was used instead of AJH. Hormone solvent routinely was mineral oil (USP), and some controls for all hormone treatments were injected (100 μl Hamilton microsyringe with a 30-ga needle) only with suitable volumes of that solvent. In most experiments 100 μg AJH in 10 μl mineral oil was injected. Neck ligature has been described (Herman, 1975b); ligated animals were maintained by daily injections of 100 μl sterile 10% glucose.

Student's t test, with statistical significance considered to be the $p = 0.05$ level or better, was used in statistical analysis. Unless otherwise noted, data are presented as mean ± standard error of the mean (SEM) and n indicates the smallest number of individuals examined.

RESULTS

Response of JH-sensitive female organs to the LD25 regimen

Effects of storage at LD25 for 5, 10, and 20 days were evaluated using several hundred newly emerged (June–August) and wild-caught (September–March) female monarchs. Except for the March females, the animals probably contained no mature oocytes (MO) when placed in the regimen (Herman, unpublished), and had ovarian (OV) and colleterial gland (CG) wet weights not significantly different from 2.0 mg and 0.9 mg, respectively, when placed at LD25 (Herman, unpublished). Of 17 females examined on arrival in the laboratory in early March only one contained MO, and mean OV and CG weights were 6.0 mg and 1.3 mg, respectively (Herman, unpublished). Thus in females placed in the LD25 regimen, the responses reported were not normally influenced by significant variations in MO or in OV or CG weights before incubation. All the June–August females were virgins; some captured in September–March had mated.

Figure 1 summarizes the production of MO at LD25 from June–March. For each storage duration the general pattern of change was comparable, *i.e.*, pronounced MO production in June–August, conspicuous depression of such activity from September–November, a rise in December to levels below those of June–August, a return to June–August values by January, and the highest mean responses in February–March. In addition, during June–August and January–March, 97% of females examined at all three storage durations contained MO, while in September–December only 50% of the females dissected had produced MO. The lowest MO production was noted in September–October, when only 23% of the females examined contained MO and no MO were found in animals incubated for 5 days. The only apparently aberrant point in Figure 1 was that obtained at 20 days in November. From the 5- and 10-day data for that month (and the trend for other months) it appears that the November 20-day value was unreasonably low, *i.e.*, not significantly higher than the 10-day value.

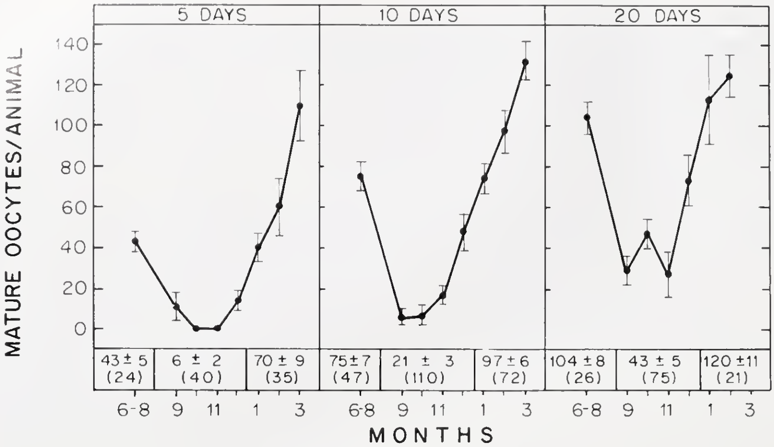


FIGURE 1. Mean mature oocyte production by monarch butterflies held at LD25 for 5–20 days. Combined mean values for June–August, September–December, and January–March, with n in parentheses, are at the bottom of the figure; months indicated by number; vertical bars indicate SEM.

Similar variations at all three storage durations occurred for both OV and CG wet weights (see Table I); *i.e.*, both organs were large in June–August, significantly smaller in September–December, and increased significantly in weight in January–March.

MO production and OV and CG development at LD25 seemed to be correlated with mating status during January–March but not September–December. As shown in Table II, organ weights in mated and virgin females did not differ significantly after storage duration in September–December. Similarly, although MO production was apparently twice as high in mated females at 10 days in September–December, that relationship was not evident at 5 or 20 days. By contrast, in mated females

TABLE I

Female monarch ovarian and colleterial gland development at LD25. Data expressed as mean wet weight in mg ± SEM; n, in parentheses, are the same for both organs; ovary (OV) values are weights of single ovaries; CG = colleterial glands; days indicate duration of storage at LD25.

	Months of arrival in laboratory		
	June–August	September–December	January–March
CG Weight			
5 days	4.9 ± 0.4 (24)	1.6 ± 0.2 (40)	5.0 ± 0.4 (35)
10 days	7.4 ± 0.4 (47)	2.7 ± 0.2 (110)	7.1 ± 0.3 (72)
20 days	8.8 ± 0.7 (26)	4.2 ± 0.3 (75)	7.8 ± 0.7 (21)
OV Weight			
5 days	28.9 ± 2.0	7.2 ± 1.7	34.8 ± 3.4
10 days	38.7 ± 2.5	14.2 ± 1.5	46.8 ± 2.2
20 days	48.5 ± 2.9	25.2 ± 2.0	52.8 ± 3.8

TABLE II

Ovarian and colleterial gland development in virgin and mated monarchs held at LD25. Data presented as mean ± SEM; single ovary (OV) and collaterial gland (CG) data in mg, mature oocyte (MO) data in total number/female; n in parentheses is same for MO, OV, and CG.

	Months of arrival in laboratory			
	September–December		January–March	
	Mated	Virgin	Mated	Virgin
OV Weight				
5 days	4.4 ± 1.0 (10)	8.1 ± 1.6 (30)	34.0 ± 4.0 (11)	21.3 ± 3.8 (12)
10 days	15.4 ± 3.3 (34)	13.8 ± 1.6 (76)	53.1 ± 2.7 (38)	37.9 ± 2.8 (27)
20 days	26.5 ± 3.7 (23)	24.4 ± 2.3 (52)	61.3 ± 5.0 (6)	49.3 ± 4.6 (13)
MO Production				
5 days	0	7 ± 3	89 ± 11	34 ± 10
10 days	32 ± 8	16 ± 3	119 ± 7	69 ± 7
20 days	46 ± 9	42 ± 6	153 ± 14	107 ± 13
CG Weight				
5 days	1.4 ± 0.2	1.7 ± 0.2	4.7 ± 0.5	3.1 ± 0.3
10 days	3.3 ± 0.4	2.5 ± 0.2	7.6 ± 0.4	6.2 ± 0.5
20 days	4.1 ± 0.7	4.3 ± 0.4	8.4 ± 1.4	7.6 ± 0.8

held at LD25 during January–March, OV and CG weights, and MO production, were always significantly higher after 5 or 10 days, and MO production was also clearly elevated after 20 days. However, mated female OV and CG weights at 20 days in January–March were not significantly higher than those of virgins. This may be due to the absence of data on March females held for 20 days. March females (usually all mated; Herman, unpublished), would probably have very high OV and CG weights and contain numerous MO after 20 days at LD25 (Figure 1). Therefore mating was correlated with enhanced JH-sensitive organ development at LD25 in January–March animals, but not in females examined during September–December. In addition, virgin MO production and OV weight in females from CA during January–March were not significantly different from those of June–August MN females, which were virgins (see Figure 1). CG weights from January–March virgins were, however, less than those of June–August at each storage duration.

From the above, it appears that adult reproductive diapause, defined as a period of significant reduction of the response of the JH-sensitive reproductive organs to LD25, lasts from September–December in the female monarch.

The response of JH-sensitive male organs to LD25

Experiments conducted simultaneously with those reported above for females, examined the responses of over 400 males stored at LD25 for 5, 10, and 20 days. Again, both newly emerged (June–August) and wild-caught (September–March) animals were used. The pattern of development of the male accessory gland (AG), tubular gland (TG), and ejaculatory ducts (ED) were all similar.

Figure 2 presents the summarized data obtained for the ED. ED weights were

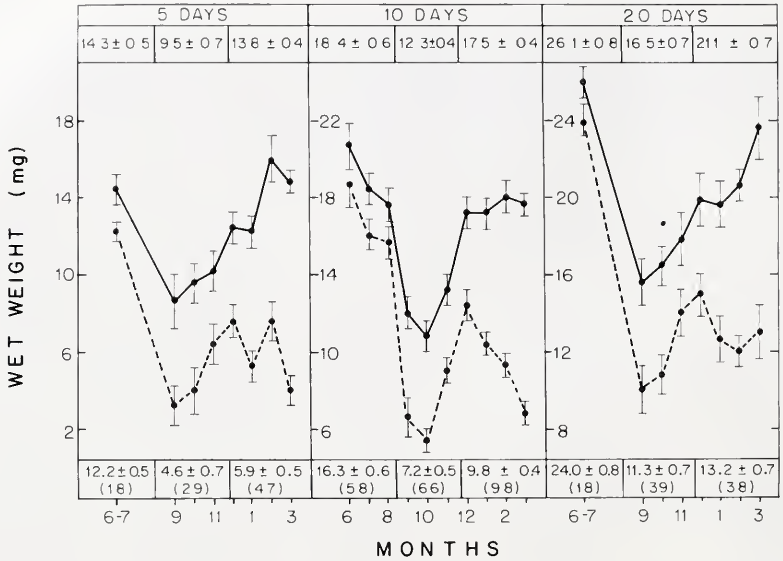


FIGURE 2. Solid and dashed lines, respectively, indicate mean gross and net changes in ejaculatory-duct wet weights in monarch butterflies held at LD25 for 5–20 days. Combined mean values for June–August, September–November, and December–March are at the top (gross changes) and bottom (net changes) of the figure; n in parentheses are the same for both gross and net responses; months indicated by number; vertical bars indicate SEM.

high in June–August. These glands' 5-day values, and those for 20-days, were nearly identical in June–July, and are combined in Figure 2. August data at 5 and 20 days were not obtained. The August mean at 10 days was significantly lower than that of June, but not July, and that difference is indicated in Figure 2. ED development declined at all three storage durations, to an apparent nadir in September–October, and was followed by a slight increase in November. The December ED mean at 10 days was not significantly different from that in July–August, and the December ED means for all three storage durations were significantly higher than those determined from the combined September–November data. ED development was erratic after December: The 5-day response was significantly elevated in February–March, the 10-day response remained unchanged, and the 20-day response increased significantly only in March.

Consideration of the response of the three male glands to LD25 was complicated by changes in the initial weights of all three glands during the annual adult cycle. Thus, at eclosion the mean wet weights of the AG, TG, and ED were, respectively, 0.9 mg, 1.6 mg, and 2.0 mg, and all June–August data were obtained from males placed at LD25 on the day of eclosion. By contrast, mean AG, TG, and ED wet weights upon butterflies' arrival in the laboratory in November were 1.3 mg, 2.0 mg, and 3.8 mg, respectively, while in March the corresponding mean values were 3.6 mg, 6.5 mg, and 10.7 mg. Therefore, the mean *net* response was examined (*i.e.*, weights after LD25 storage minus mean weight on arrival in the laboratory) for all three glands in each month. The calculations yielded qualitatively comparable response patterns for all three male glands. They are illustrated in Figure 2 by the dashed-line curves obtained for the ED net response.

Net ED responses were highest in June–August, fell to low levels in September–

November, and seemed to rise to a post-November peak in December. Net ED means in January–March, with the single exception of the 5-day February values, were consistently lower than those seen in December; *i.e.*, net response apparently declined after December. In addition, although the December–March net weight means at all storage durations were higher than those of September–November, they were only significantly higher in males stored at LD25 for 10 days. It therefore appears that while gross ED response in December–March approached that found in June–August, net ED response did not increase substantially after September–November and never reached a level comparable to that observed in June–August.

The gross and net responses of the AG and TG are summarized in Table III. The general pattern of mean gross and net development for both glands was the same as that illustrated more fully for the ED in Figure 2. However, the AG were the only male glands in which the gross and net increases in December–March were all significantly higher in September–November, and in which the 5- and 10-day gross values in December–March were significantly higher than those observed in June–August.

The above data indicate that an adult reproductive diapause occurs from September–November in male Monarch butterflies.

Comparison of the male and female responses to LD25

To compare the responses to LD25 of the JH-sensitive male and female organs during diapause and postdiapause periods, mean values for MO production and

TABLE III

Effect of LD25 environment on development of male monarch accessory and tubular glands. Data presented as mean mg wet weight ± SEM; n, in parentheses, is same for both glands; days refer to time held at LD25; net = weight after LD25 storage (i.e., gross weight) minus mean weight of same glands upon arrival in the laboratory.

	Months of arrival in laboratory		
	June–August	September–November	December–March
Accessory glands			
5 days—gross	4.4 ± 0.2	3.3 ± 0.2	5.2 ± 0.2
net	3.5 ± 0.2	1.8 ± 0.3	2.6 ± 0.2
	(18)	(29)	(47)
10 days—gross	6.3 ± 0.2	4.7 ± 0.2	7.2 ± 0.2
net	5.4 ± 0.2	3.2 ± 0.2	4.6 ± 0.2
	(59)	(66)	(98)
20 days—gross	10.9 ± 0.5	7.3 ± 0.3	9.2 ± 0.3
net	10.0 ± 0.5	5.7 ± 0.3	6.6 ± 0.3
	(20)	(37)	(38)
Tubular glands			
5 days—gross	12.0 ± 0.5	5.5 ± 0.4	9.0 ± 0.4
net	10.4 ± 0.5	2.8 ± 0.5	4.2 ± 0.3
10 days—gross	12.7 ± 0.6	7.5 ± 0.3	10.9 ± 0.3
net	11.0 ± 0.4	4.7 ± 0.3	6.2 ± 0.3
20 days—gross	17.2 ± 0.6	10.2 ± 0.4	13.3 ± 0.5
net	15.6 ± 0.6	7.4 ± 0.4	8.5 ± 0.5

male and female organ wet weights obtained at 5, 10, and 20 days were converted to percentages of the pertinent mean values at the same storage durations in June–July. Means of these individual percentages were used to estimate the changes in the responses of the pertinent organs (Table IV).

The diapause (September–December) female response was collectively about one-third of that in prediapause (June–August), while the diapause (September–November) male response was about two-thirds of that seen in prediapause. In addition, the postdiapause (January–March) female response was collectively 83% higher than that observed in diapause, but the postdiapause (December–March) male response was only 23% higher than that seen in diapause. The above differences would be even more pronounced if net, rather than gross, male response had been used for comparison. Similarly, 20-day March female data would presumably accentuate these sexual differences. Thus, development of the JH-sensitive female organs is more depressed during diapause, and more enhanced during postdiapause, than is that of the JH-sensitive male organs.

When individual male and female organs were considered, it appeared that MO production was the single event most profoundly influenced during diapause and postdiapause; it exhibited the lowest percentage response to each storage duration during diapause and the highest percentage response at each storage duration during postdiapause. The means obtained for MO percent response, however, were not significantly different from those obtained for the OV and CG responses during either diapause or postdiapause. In males, the AG and ED had statistically equivalent responses in both periods, and the responses of these two organs were significantly different from those of the TG. Postdiapause AG and ED development, based upon gross response percentages, was not significantly different from that of the female CG, but this apparent similarity was eliminated by consideration of net responses.

Figure 3 shows that after 5 days at LD25, MO production in diapause females was only about 14% and 9% of that found in pre- and postdiapause females, respectively. By contrast, in females held at LD25 from 5 to 20 days, mean MO production increased 720% (6–43 MO) in diapause monarchs, but only 240% and

Table IV

Comparison of responses of JH-sensitive male and female monarch reproductive organs during diapause and postdiapause. Data expressed as mean ± SEM; raw data used to determine percentages presented in Figs. 1 and 2 and Tables 1 and 3; numbers in parentheses refer to number of percentages available for determining each mean value; abbreviations presented in earlier figures and tables.

	Percent of June–July mean response	
	Diapause	Postdiapause
Female		
MO production (3)	28 ± 7	136 ± 7
OV weight (3)	38 ± 6	116 ± 4
CG weight (3)	39 ± 4	96 ± 3
All above (9)	35 ± 4	118 ± 7
Male		
AG weight (3)	70 ± 2	102 ± 8
TG weight (3)	53 ± 3	77 ± 1
ED weight (3)	66 ± 1	90 ± 4
All above (9)	63 ± 3	86 ± 5

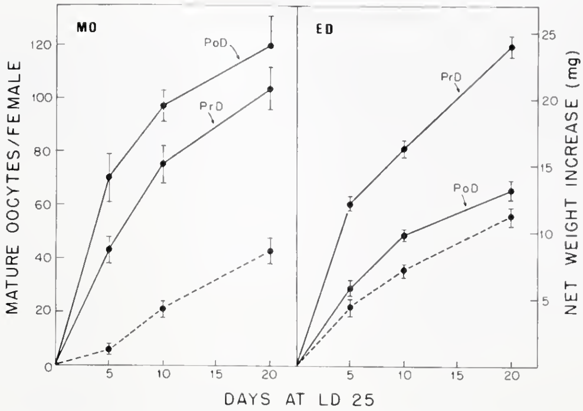


FIGURE 3. Mean mature oocyte production (MO) and ejaculatory duct (ED) net wet weight changes in monarch butterflies held at LD25 for various intervals. Dashed line indicates diapause animals, and solid lines indicate prediapause (PrD) and postdiapause (PoD); vertical bars indicate SEM. Numbers of animals tested same as in Figures 1 and 2.

170% in pre- and postdiapause animals, respectively. Comparable, but quantitatively less striking changes occurred in the male ED (Figure 3). After 5 days at LD25 the mean net wet weight increase of these organs in diapause males was 37% and 78% that observed in pre- and postdiapause males, respectively. By contrast, the mean net wet weight increase of the ED in diapause males held at LD25 for 5 to 20 days was 246% (4.6–11.3 mg), while the comparable values for pre- and postdiapause males were 107% and 224%, respectively. (Males differed from females in that weight changes in the postdiapause ED more closely resembled those of diapause animals.) Comparable plots of the OV and CG data of Table I, and the AG and TG data of Table III, produced similar results. Thus, monarch reproductive diapause was characterized by a severe depression of JH-sensitive organ development during the first 5 days at LD25. Storage of monarchs at LD25 from 5 to 20 days, however, results in a developmental rate of the pertinent organs during diapause that apparently exceeds that observed in prediapause and postdiapause.

Effect of juvenile hormone on diapause and postdiapause monarchs

Diapause males and females were fed immediately after arrival in the laboratory, injected with 100 μg AJH or used as controls, held at LD25 for 10 days, and then dissected. Comparable experiments were performed in each of 3 diapause months (i.e., September–November) in each of 2 years. The results of individual experiments, and the combined data (see Table V,a), show that diapause monarchs of both sexes responded to AJH.

Less extensive experiments examined the response of neck-ligated diapause (October) and postdiapause (February) monarchs to JH injections. Animals were fed, neck-ligated, and then injected with either mineral oil or 10 μg JH I, immediately after arrival in the laboratory. They were then held at LD25 for 6 days. The results (see Table V,b) indicated significantly greater postdiapause responses in all JH-sensitive organs in both sexes.

The above figures reflect net changes for females, since there were no major differences in the OV and CG weights obtained from diapause and postdiapause female controls and no female control produced any MO. However, the gross data

TABLE V

Effects of juvenile hormone on intact and neck-ligated male and female monarchs. Data presented as mean wet weight (mg) or mean total MO production ± SEM; organ abbreviations as in previous tables and figures; n, in parentheses, is same for all male or female values; controls uninjected and mineral oil injected; diapause animals captured in September–November and postdiapause animals captured in February.

	(a) Intact diapausers		(b) Neck-ligated + 10 μg JH I	
	Control	100 μg AJH	Diapause	Postdiapause
Female	(32)	(45)	(8)	(9)
MO production	12 ± 3	91 ± 9	56 ± 17	143 ± 15
OV weight	12.5 ± 1.9	41.1 ± 3.2	24.0 ± 4.9	49.7 ± 4.4
CG weight	2.3 ± 0.3	5.6 ± 0.4	5.3 ± 0.7	7.5 ± 0.8
Male	(31)	(34)	(5)	(9)
AG weight	5.7 ± 0.2	7.3 ± 0.4	3.6 ± 0.5	5.3 ± 0.3
TG weight	8.8 ± 0.4	11.2 ± 0.5	6.2 ± 0.7	8.8 ± 0.6
ED weight	14.1 ± 0.5	18.1 ± 0.7	11.2 ± 1.3	14.8 ± 0.8

are misleading with regard to males. As mentioned earlier, postdiapause male glands on arrival in the laboratory were significantly heavier than those of diapause males, and the postdiapause male controls in this experiment were significantly larger than those of diapause controls. Thus control diapause males (n = 17) had weights of 1.3 ± 0.1 mg, 2.4 ± 0.3 mg, and 4.0 ± 0.4 mg for the AG, TG, and ED, respectively, while the corresponding values for eight postdiapause male controls were 3.1 ± 0.3 mg, 7.0 ± 1.0 mg, and 10.7 ± 1.2 mg, respectively. The mean net response to JH I showed the AG, TG, and ED were 2.3 ± 0.5 mg, 3.8 ± 0.7 mg, and 7.1 ± 1.3 mg, respectively, in diapause males, but only 2.2 ± 0.3 mg, 1.8 ± 0.6 mg, and 4.1 ± 0.8 mg, respectively, in postdiapause males. Therefore, it appears that the gross response of the postdiapause male glands to JH I is significantly higher than that of diapause males, but the net response is either comparable (AG) or significantly less (TG and ED).

Effect of natural environment on diapause monarchs

Other experiments examined the response of wild-caught September monarchs to storage in natural (*i.e.*, outdoors) conditions. In these studies, monarchs captured during early September were either dissected immediately or held outdoors on a porch adjacent to the laboratory for 5–7 days. Some animals of each sex were uninjected, while others were injected with hormone solvent or 100 μg AJH.

In initial experiments all control and AJH injected animals were fed both after capture and daily during the storage period. All of these animals exhibited significant increases in body weight; *e.g.*, 26 males on arrival in the laboratory weighed 465 ± 16 mg, while 35 control males (fed daily) weighed 689 ± 18 mg after 5–7 days outdoors. In addition, even though AJH injections significantly elevated all pertinent organ weights of both sexes over control values, all JH-sensitive organs of both male and female controls were significantly heavier than those of animals dissected immediately after capture; *e.g.*, ED values were 5.3 ± 1.0 mg, 8.2 ± 0.5 mg, and 11.7 ± 0.4 mg for 26 on arrival, 35 control, and 24 AJH-injected males, respectively. The elevation of control over arrival values was not anticipated, since it was expected that the outdoor environment would not promote development of JH-sensitive organs. However, the results suggested that daily feeding, and the associated increases in body weights, might promote growth of JH-sensitive organs. Therefore, animals fed only after capture and once or twice during the storage period were studied. In these, there were no deaths, and no significant body weight changes in control or AJH injected animals. Furthermore, control organs were identical in wet weight to those found on arrival, and AJH injections again led to significant increases in all pertinent organs; *e.g.*, CG weights were 0.8 ± 0.1 mg, 0.8 ± 0.1 mg, and 3.2 ± 0.5 mg in 32 on-arrival, 17 control, and 7 AJH-injected females, respectively. Owing to the above findings all data dealing with the effects of storage of diapause monarchs in the outdoor environment were converted to mg wet weight (or MO) per gm body weight (Table VI).

When corrected for body weight variations, the data showed no change in any JH-sensitive male or female organ of control animals during 5–7 days storage in natural conditions. (By contrast, both male and female organs developed markedly when newly emerged July monarchs were held outdoors for similar periods, *e.g.*, male ED weighed 23.5 ± 1.3 mg and female OV weight was 33.5 ± 4.2 mg.) Both monarch sexes exhibited significant weight increases in all JH-sensitive organs after AJH injections. MO were found in only one of 33 females on arrival, and only two of 39 control females, while 23 of 33 females treated with AJH contained MO. Thus, diapause male and female monarchs held in their natural environment responded to AJH, but did not exhibit significant development of JH-sensitive organs in the absence of exogenous hormone.

Alterations in organs lacking sensitivity to juvenile hormone

JH regulation of the male testes and seminal vesicle-vas deferans complex, or the female bursa copulatrix and receptacle gland has not been demonstrated (Herman, 1975a, b; Johnson, 1979). Observations on these four organs during the investigations described above revealed that patterns of development were not comparable to those noted for JH-sensitive male and female reproductive organs. None

TABLE VI

Effects of outdoors environment, with or without juvenile hormone injections, on wild-caught diapause monarchs in September. Data presented as mean mg wet weight (or MO)/gm body weight ± SEM; abbreviations as in previous tables and figures; n in parentheses; experiments conducted in September; all animals held in outdoors September environment for 5–7 days; controls either uninjected or injected with mineral oil.

	AG	TG	ED
Males			
On arrival (26)	4.3 ± 0.8	7.4 ± 1.5	12.3 ± 2.4
Controls (50)	4.4 ± 0.3	7.3 ± 0.7	12.5 ± 1.1
100 μg AJH (31)	6.2 ± 0.4	11.6 ± 0.8	18.1 ± 1.1
	MO	OV	CG
Females			
On arrival (33)	5 ± 5	8.8 ± 4.0	2.2 ± 0.5
Controls (39)	2 ± 2	6.8 ± 1.4	2.0 ± 0.2
100 μg AJH (33)	42 ± 9	31.3 ± 3.7	6.6 ± 0.6

of these four organs exhibited clearly depressed responses during diapause coupled with significantly elevated weights during postdiapause when intact animals were held for 5, 10 (see Table VII), or 20 days at LD25. In addition, when diapause monarchs held outdoors were injected with AJH, only the receptacle gland showed significant enlargements. In similarly treated diapause animals held at LD25, only the testes apparently responded to AJH (Table VII). Since these four organs showed no selective depression of response to LD25 during diapause, and no consistent responses to AJH injections, it appears that they are all controlled by regulatory mechanisms different from those influencing the JH-sensitive male and female reproductive organs.

Effects of SD10 storage on June–July animals

Effects of SD10 storage on monarch reproductive tract development at SD10 and to LD25 after various periods of SD10 storage were studied. Monarchs emerging in the laboratory during June–July were placed at SD10 on the day of eclosion and held there for up to 5 months, with weekly feeding. Several animals of each sex were removed from SD10 at 30-day intervals and either dissected immediately or fed and placed at LD25 for 10 days, with daily feeding, prior to dissection.

The male reproductive glands enlarged continuously during the SD10 storage period. Thus, eight males held for 30 days at SD10 possessed AG, TG, and ED with wet weights of, respectively, 1.2 ± 0.1 mg, 2.6 ± 0.4 mg, and 5.2 ± 0.8 mg, while four males held for 150 days had AG, TG, and ED weighing 2.8 ± 0.4 mg, 6.9 ± 1.5 mg, and 18.0 ± 4.5 mg, respectively. The 150-day value for the ED was not significantly different from that of June–July animals held for 10 days at LD25 immediately after eclosion. The data from all males held at SD10 for 1–5 months is summarized in Table VIII. Slight but significant enlargements were noted at 30 days for the female OV and CG, but further changes were not observed in females stored at SD10 for 60–150 days, and no female held at SD10 produced any MO.

TABLE VII

Effects of various treatments on monarch reproductive organs lacking sensitivity to juvenile hormone. Data presented as mean mg wet weight ± SEM; LD25 animals held for 10 days; SV-VD, BC, and RG are seminal vesicles-vas deferans, bursa copulatrix, and receptacle gland, respectively; reproductives from June–August, diapause-LD25 animals from September–November (males), or September–December (females); diapause-outdoors from September; and postdiapause animals from December–March (males) or January–March (females); about half controls given 10 μl mineral oil.

	Males			Females		
	Testes	n	SV-VD	BC	n	RG
LD25						
Reproductive	12.2 ± 0.3	59	12.0 ± 0.3	13.6 ± 0.5	47	2.1 ± 0.1
Diapause	8.7 ± 0.2	79	14.4 ± 0.4	13.2 ± 0.7	112	1.9 ± 0.0
Postdiapause	8.9 ± 0.2	98	14.6 ± 0.2	15.9 ± 2.2	72	1.5 ± 0.0
Diapause-outdoors						
Controls	10.4 ± 0.3	35	10.6 ± 0.4	11.5 ± 0.5	43	2.6 ± 0.1
100 μg AJH	11.0 ± 0.4	24	10.8 ± 0.6	11.3 ± 0.5	26	3.0 ± 0.1
Diapause-LD25						
Controls	9.0 ± 0.2	31	14.4 ± 0.5	11.9 ± 0.3	31	1.9 ± 0.1
100 μg AJH	9.8 ± 0.3	33	14.3 ± 0.6	12.6 ± 0.4	27	1.6 ± 0.1

TABLE VIII

Effect of SD10 storage on development of monarch reproductive organs and their subsequent response to LD25. Data presented as mean mg wet weight (or MO) ± SEM; SD10 only = held at SD10 for 30–150 days; LD25 only = held at LD25 for 10 days after eclosion; SD10 + LD25 = SD10 for 30–150 days + 10 days at LD25; see text for other abbreviations; n in parentheses.

	AG	TG	ED
Males			
At eclosion (35)	0.9 ± 0.0	1.6 ± 0.1	2.1 ± 0.1
SD10 only (37)	2.0 ± 0.1	5.2 ± 0.4	12.2 ± 1.0
LD25 only (28)	7.0 ± 0.3	13.8 ± 0.5	19.2 ± 0.7
SD10 + LD25 (33)	7.1 ± 0.2	13.4 ± 0.9	21.8 ± 1.0
	MO	OV	CG
Females			
At eclosion (16)	0	1.9 ± 0.1	0.8 ± 0.0
SD10 only (34)	0	3.3 ± 0.2	1.1 ± 0.1
LD25 only (29)	72 ± 9	38.3 ± 3.3	7.4 ± 0.5
SD10 + LD25 (36)	93 ± 9	41.2 ± 6.6	7.8 ± 0.4

These studies also showed that storage at SD10 for any duration was without apparent effect on subsequent response to LD25. There was no indication of a diapause-like response in any male or female monarch held at SD10 for any length of time, and all but one female placed at LD25 after SD10 storage produced MO. Thus, the mean values from combined results (see Table VIII) from all males and females held at LD25 after storage at SD10 for 30–150 days did not differ significantly from results from June–July monarchs placed at LD25 on the day of emergence.

DISCUSSION

The above experiments demonstrate an adult reproductive diapause, defined as a period of significant reduction in the response of certain reproductive organs to summer-like (LD25) conditions, in both male and female monarch butterflies. This period of reduced response lasts from September–November in males, and from September–December in females. It can be easily demonstrated, in all male and female organs known to be sensitive to JH, by holding monarchs collected in the wild at monthly intervals at LD25 for 5, 10, or 20 days. As in many other insects (Tauber and Tauber, 1976), the adult monarch reproductive diapause is characterized by: (1) beginning when environmental conditions might still permit some successful reproduction (*i.e.*, late August to early September in MN); (2) a period of diminishing diapause intensity (*i.e.*, after October in the overwintering colonies); and (3) ending before the return of environmental conditions that promote full reproductive activity in both sexes (*i.e.*, in November or December in the overwintering colonies).

The above experiments seem to document a monarch butterfly adult diapause and its duration. However, information is lacking on its induction, maintenance, and termination, and the relative roles of photophase and temperature. The available data indicate that storing newly emerged animals in constant, suboptimal environments does not induce diapause in adult monarchs. In this report, holding new adults at SD10 for up to 5 months did not result in a diapause-like response to LD25. Similarly, earlier studies (Barker and Herman, 1976) have shown that monarch females emerging in June–July, and held on short photophases and relatively low temperatures, still produce more MO than are found in diapause females on arrival in the laboratory. Therefore, although adult monarchs are sensitive to both photoperiod and temperature, adult reproductive diapause may be programmed in some earlier developmental stage.

The data of Tables V and VI, and earlier studies (Pan and Wyatt, 1971; Barker and Herman, 1973, 1976; Herman, 1973, 1975a, b; Herman and Barker, 1977), strongly suggest that effective JH hemolymph titers are depressed during monarch reproductive diapause. Thus, animals of both sexes held at LD25 exhibit low levels of JH-sensitive organ development, but such development is enhanced by injections of AJH. Similarly, JH-sensitive organs did not grow in diapause animals held in the natural environment in September, but such growth is induced in both males and females by JH treatment. Furthermore, hemolymph JH titers in wild-caught monarchs of both sexes are lower in diapause than in the reproductively active months of June–July (Lessman, 1980). Therefore, it is likely that effective JH hemolymph titers during monarch reproductive diapause are significantly less than those during periods of reproductive activity. Whether this reduction of effective JH titer is due to decreased JH biosynthesis, enhanced JH inactivation, both processes, or some other factor(s) remains to be determined. Reduction of JH-sensitive male and female organ response to JH I during diapause (see Table V,b), suggests enhanced JH inactivation, or decreased target tissue sensitivity, during the diapause.

Comparison of results from male and female monarchs demonstrates differences in diapause and postdiapause development of JH-sensitive organs. The prediapause response level was first reached in December in males but not until January in females held at LD25. Similarly, the response of female JH-sensitive organs to LD25 storage during diapause was only 35 ± 4% of that observed in June-July while the comparable percentage for the male diapause response was 63 ± 3%. Therefore, female diapause is both longer and more intense than that of males. Postdiapause sexual differences were also observed. After arrival in the laboratory, female JH-sensitive organs remained substantially unaltered for most of the postdiapause period (Herman, unpublished), and the response of these organs to LD25 storage typically met or exceeded the prediapause values. By contrast, JH-sensitive male organs exhibited progressive enlargements (similar to those noted at SD10) on arrival in the laboratory. Furthermore, the gross responses of the pertinent male organs to LD25 storage rarely equalled those of prediapause animals, and the net responses were typically not significantly higher than those observed during diapause. Apparently, postdiapause female (but not male) development in the natural environment is repressed, and the postdiapause female (but not the male) retains the ability to respond to LD25 in a manner comparable to that of the prediapause animal.

Studies on neck-ligated diapause monarchs show that males are more sensitive to JH I, JH II, and AJH than are females, *e.g.*, the average effective dose (ED_{50}) for AJH effects on the pertinent organs was 49 μg in males and 253 μg in females (Lessman, 1980). Similarly, posteclosion female monarchs routinely reach a peak hemolymph JH titer of about 10^6 *Galleria* units/ml before the onset of rapid oogenesis, while male values during rapid reproductive gland growth average about 10^4 *Galleria* units/ml (Lessman, 1980). Unpublished experiments in my laboratory show an absence of female tract development, but significant male reproductive gland growth, in allatectomized animals held at LD25 for several weeks. Apparently, male reproductive glands can exhibit substantial growth when hemolymph JH is low or absent, and they can also be stimulated by JH levels ineffective in females. Thus the shorter and less intense male diapause, and the development of the male tract in normal environmental conditions during postdiapause, may be due to either male (but not female) sensitivity to low hemolymph JH titers, or to male (but not female) tract development in the absence of JH.

Mating stimulates both oogenesis and oviposition in reproductively active summer monarchs, and the available evidence suggests that enhanced oocyte production in mated females is associated with elevated JH titers (Herman and Barker, 1977). A comparable situation does not exist in monarch females captured in the wild from September–February, since about 50% of the females examined during that period were mated but practically none contained mature oocytes on arrival (Herman, unpublished). Similarly, prior mating did not stimulate the female reproductive tract in monarch females held at LD25 during diapause. By contrast, mated postdiapause females had significantly larger OV and CG, and produced more MO, than did virgins after LD25 storage. Apparently, mating elevates effective JH titers (as measured by the growth of JH-sensitive female organs) in both reproductive and postdiapause, but not diapause, females held at LD25. It would be interesting to know more about the mechanism(s) by which mating influences effective JH titers, and the alterations in this mechanism during overwintering.

Adult diapause might facilitate survival of the overwintering monarch population. Existing data indicate that monarch diapause only occurs in adults emerging in late August or September; there is no reason to believe that individual adults that have previously been reproductively active can subsequently become diapause monarchs (Barker and Herman, 1976; Herman, unpublished). When the diapause generation begins to emerge there are still many flowering plants blooming, and thus many sources of nectar. Field observations document that feeding is a major activity of premigratory diapause adults. Adult diapause also begins in the Minneapolis area about 1 month before the first frost. Field observations show that milkweed plants are rapidly deteriorating during this period. Since rearing data (Urquhart, 1960; Herman, unpublished) indicate that oviposition to adult emergence usually takes 3–4 weeks outdoors in June–August, it appears that the cessation of reproduction associated with diapause prevents a wasteful expenditure of reserves that would probably yield few if any viable adults. Conserving protein, especially, might be important to monarchs, since their reproduction entails substantial loss of fat body protein (Herman, unpublished). Since mating is rare in the premigratory population (Herman, unpublished), bursa copulatrix weights are low in the absence of spermatophores and male reserves are not depleted by production and export of spermatophores. Similarly, the possibility of mating-induced oogenesis and oviposition, like that observed in summer reproductives (Herman and Barker, 1977), is reduced. Thus, diapause initiation apparently results in a young monarch population with high levels of nutrient reserves, unencumbered by well-developed reproductive tracts, and hence highly suited for the long migratory flights characteristic of the species (Brower, 1977).

A "diapause break" during migration is unlikely, since migratory activity (Urquhart, 1960; Brower, 1977) and diapause both reach peaks in September–October. Furthermore, migratory flight may speed up JH degradation (Lessman, 1979) and effectively decrease hemolymph JH levels. Thus, both reproductive diapause intensity and flight-enhanced JH hydrolysis would inhibit futile reproductive activity and the resultant loss of stored reserves. In addition, the lipid accumulated during the premigratory period of reproductive diapause (Brown and Chippendale, 1974) and presumably mobilized by the adipokinetic hormone for flight fuel (Dallmann and Herman, 1978), could be replenished indirectly by nectar feeding during migration. Thus, most monarchs probably would arrive at the overwintering colonies in excellent condition to withstand the coming winter. The data available supports this expectation (Tuskes and Brower, 1977).

After the overwintering colonies form, female reproductive diapause would last into December, when suboptimal environments could ensure suppression of female reproductive activity (Barker and Herman, 1976). Similarly, females' longer diapause might enhance their ability to withstand the rigors of winter, since diapause is frequently characterized by reduced metabolic activity (Tauber and Tauber, 1976). The earlier end of diapause in males, and the ensuing increase in male gland development and mating behavior in environments that completely suppress female tract development (Herman, unpublished), could transfer nutrients (especially protein?) from males to females, as it appears to do in reproductively active summer monarchs (Boggs and Gilbert, 1979). If so, such a mechanism could partly explain the ability of postdiapause females, even though they are already 5–6 months old, to produce oocytes at a rate at or above that of summer reproductives. Transfer of nutrients and sperm simultaneously also could help males retain their genes in the monarch population. This line of reasoning implies a decline in male, but not female, condition during the postdiapause period. Evidence for such a decline comes from the developmental pattern of male glands at LD25 during the postdiapause period. In addition, a decline in male vigor during overwintering is indicated by data on aging showing that at LD25 postdiapause males do not live as long as postdiapause females (Herman, unpublished). Thus, the available evidence, although inconclusive, implies that postdiapause male mating activity in the overwintering colony may significantly enhance female's survival and fecundity. It further suggests that many males may normally be sacrificed to insure survival of the monarch population. The result of all diapause and postdiapause activities in both sexes would be a population of previously mated, but nutrient poor (or dead), males and a population of relatively well-nourished, and mated, females with no substantial decrease in life expectancy. Such a population would seem to be admirably suited to re-establish the reproductive generations. It might also account for the fact that most females, but far fewer males, emigrate from the overwintering colonies (Williams *et al.*, 1942; Herman, unpublished). Since many of the above speculations can be tested experimentally, it will be of interest to see if future research confirms or refutes some or all of these ideas.

These studies have dealt with monarchs captured or reared in MN from June–September, and with monarchs captured in CA from October–March. However, monarchs from the eastern United States typically overwinter in Mexico, while CA monarchs rarely migrate into the eastern portion of the country (Urquhart, 1960; Urquhart and Urquhart, 1978; Brower, 1977). This population separation suggests the possibility of physiological differences between the two groups of butterflies. Data indicating geographical differences in diapause of other insect species has been presented (Tauber and Tauber, 1976). However, to my knowledge no data substantiates such differences in monarchs. The findings of Tables I and II indicate no obvious differences in eastern September and western October monarchs. Similarly, the fact that western postdiapause virgins produce oocytes at rates comparable to those found in eastern prediapause virgins argues for comparable activities in the two populations. Thus, the above discussion has assumed that overwintering monarchs in Mexico and CA, and reproductively active monarchs in both the eastern and western United States, are in similar physiological states. It will be of interest to see if future studies on western reproductively active, and overwintering Mexican, monarchs support this assumption.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to those who assisted in the capture, rearing, and feeding of the monarchs used in these studies. In this regard, special thanks are due to Mr. Paul Cherubini, Mr. and Mrs. David Preus, and Alex, Max, Carter, and especially Charlotte Herman. I also deeply appreciate the efforts of those residents of Minnesota, particularly Mrs. Dorothy Jackson, who located migratory swarms of butterflies and were kind enough to guide me to them. Dr. Louise Rollins-Smith conducted some of the dissections done in the early stages of this work, and her highly competent assistance was invaluable.

LITERATURE CITED

BARKER, J. F., AND W. S. HERMAN. 1973. On the neuroendocrine control of ovarian development in the Monarch butterfly. *J. Exp. Zool.* **183:** 1–10.

BARKER, J. F., AND W. S. HERMAN. 1976. Effect of photoperiod and temperature on reproduction of the Monarch butterfly, *Danaus plexippus. J. Insect Physiol.* **22:** 1565–1568.

BOGGS, C. L., AND L. E. GILBERT. 1979. Male contribution to egg production in butterflies: Evidence for transfer of nutrients at mating. *Science* **206:** 83–84.

BROWER, L. P. 1961. Studies on the migration of the Monarch butterfly. I. breeding populations of *Danaus plexippus* and *D. gilippus berenice* in south central Florida. *Ecology* **42:** 76–83.

BROWER, L. P. 1977. Monarch migration. Natural History **86:** 40–53.

BROWN, J. J., AND G. M. CHIPPENDALE. 1974. Migration of the Monarch butterfly, *Danaus plexippus*: energy sources. *J. Insect Physiol.* **20:** 1117–1130.

DALLMANN, S. H., AND W. S. HERMAN. 1978. Hormonal regulation of hemolymph lipid concentrations in the Monarch butterfly. *Gen. Comp. Endocrinol.* **36:** 142–150.

DORES, R. M., S. H. DALLMANN, AND W. S. HERMAN. 1979. The regulation of post-eclosion and post-feeding diuresis in the Monarch butterfly, *Danaus plexippus. J. Insect Physiol.* **25:** 895–901.

FUNK, R. S. 1968. Overwintering of Monarch butterflies as a breeding colony in southwestern Arizona. *J. Lepid. Soc.* **22:** 63–64.

HERMAN, W. S. 1973. The endocrine basis of reproductive inactivity in Monarch butterflies overwintering in central California. *J. Insect Physiol.* **19:** 1883–1887.

HERMAN, W. S. 1975a. Juvenile hormone stimulation of tubular and accessory glands in male Monarch butterflies. *Comp. Biochem. Physiol.* **51A:** 507–510.

HERMAN, W. S. 1975b. Endocrine regulation of posteclosion enlargement of the male and female reproductive glands in Monarch butterflies. *Gen. Comp. Endocrinol.* **26:** 534–540.

HERMAN, W. S., AND J. F. BARKER. 1977. Effect of mating on Monarch butterfly oogenesis. *Experientia* **33:** 688–689.

HILL, H. F., A. M. WENNER, AND P. H. WELLS. 1976. Reproductive behavior in an overwintering aggregation of Monarch butterflies. *Am. Midl. Nat.* **95:** 10–19.

JOHNSON, C. G. D. 1979. *The accessory reproductive glands of the male Monarch butterfly, Danaus plexippus: their development, fine structure, and biochemistry.* Ph.D. Thesis, University of Minnesota, St. Paul, MN. 162 pp.

LESSMAN, C. 1979. Enhancement of juvenile hormone degradation by flight and heat in adult Monarchs. *Am. Zool.* **19:** 915.

LESSMAN, C. 1980. Biology of juvenile hormone in the Monarch butterfly, *Danaus plexippus.* Ph.D. Thesis, University of Minnesota, St. Paul, MN. 156 pp.

PAN, M. L., AND WYATT, G. R. 1971. Juvenile hormone induces vitellogenin synthesis in the Monarch butterfly. *Science* **174:** 503–505.

TAUBER, M. J., AND C. A. TAUBER. 1976. Insect seasonality: diapause maintenance, termination, and postdiapause development. *Ann. Rev. Entomol.* **21:** 81–107.

TUSKES, P. M., AND L. P. BROWER. 1978. Overwintering ecology of the Monarch butterfly, *Danaus plexippus*, in California. *Ecol. Entomol.* **3:** 141–154.

URQUHART, F. A. 1960. *The Monarch Butterfly*. University of Toronto Press, Toronto, Canada. 361 pp.

URQUHART, F. A., and N. R. URQUHART. 1978. Autumnal migration routes of the eastern population of the Monarch butterfly (*Danaus p. plexippus* L.; Danaidae; Lepidoptera) in North America to the overwintering site in the neovolcanic plateau of Mexico. *Can. J. Zool.* **56:** 1759–1764.

URQUHART, F. A., N. R. URQUHART, AND F. MUNGER. 1968. A study of a continuously breeding population of *Danaus plexippus* in southern California compared to a migratory population and its significance in the study of insect movement. *J. Res. Lepid.* **7:** 169–181.

WIGGLESWORTH, V. B. 1965. *The Principles of Insect Physiology*. Methuen and Co., Ltd. London.

WILLIAMS, C. B., G. F. COCKBILL, M. E. GIBBS, AND J. A. DOWNES. 1942. Studies on the migration of Lepidoptera. *Trans. Entomol. Soc. Lond.*, **92:** 101–283.

Reference: *Biol. Bull.*, **160:** 107–113. (February, 1981)

THE ASSOCIATION BETWEEN *MYTILUS CHILENSIS* HUPE (BIVALVIA, MYTILIDAE) AND *EDOTEA MAGELLANICA* CUNNINGHAM (ISOPODA, VALVIFERA) IN SOUTHERN CHILE[1]

EDUARDO JARAMILLO, JORGE NAVARRO, AND JÜRGEN WINTER

Instituto de Zoología, Universidad Austral de Chile, Valdivia, Chile

ABSTRACT

The association between *Mytilus chilensis* and the isopod *Edotea magellanica* in two natural banks of *Mytilus chilensis* from the intertidal area of the Straits of Magellan (southern Chile) is described. This may be the first documentation of such an association between a bivalve mollusc and an isopod. In Gregorio and Santiago Bays, 18.3% and 7.6%, respectively, of the mussels investigated were occupied by isopods. The highest degree of isopod occupation was found in mussels between 30 mm and 45 mm in shell length. The regression lines for the relationship between shell length and dry-tissue weight of mussels with and without isopods showed that the isopods have no influence on the meat content of the mussels. *Edotea magellanica* is a non-obligatory commensal, nourished on food that is associated with the host's activity (formation of pseudofeces) but that is not part of the host's food. The life cycle of *Edotea magellanica* was schematically constructed using the available data. The complete life cycle of the isopod can take place inside the mantle cavity of the mussel, and in many cases two isopod generations were observed in one and the same mussel.

INTRODUCTION

Marine bivalve molluscs are well documented in the literature as hosts for symbionts. Excellent reviews on this topic have been presented by Galtsoff (1964), Cheng (1967), and Sindermann (1970). No literature available to the authors, however, reported a commensalistic/parasitic association between bivalve molluscs and an isopod.

This paper describes such an association between *Mytilus chilensis* Hupé and the isopod *Edotea magellanica* Cunningham (Valvifera, Idotheidae). This isopod was first described in 1871, but never recorded as living within the mantle cavity of bivalve molluscs. The only hint of such an association is in Schultz (1969, p. 19), Schultz assumed that isopods are perhaps also parasitic on large molluscs such as clams.

In the intertidal area of the Straits of Magellan (southern Chile), numerous natural banks of *Mytilus chilensis* exist, and *E. magellanica* is found in comparatively high densities inside the mantle cavity of this mussel. We investigated two of these natural banks to determine the amount of occupation and the kind of association between the two species.

MATERIALS AND METHODS

In August 1978, specimens of *Mytilus chilensis* were collected from Gregorio Bay (52°37′ S, 70°08′ W) and Santiago Bay (52°31′ S, 69°51′ W), on the northern

---

Received 11 July 1979; accepted 11 November 1980.

[1] This research was supported by Grant S-78-8 (1978) from the Dirección de Investigación, Universidad Austral de Chile.

side of the Straits of Magellan. The samples, taken during low tide from natural banks, included specimens from the entire intertidal area. The mussels were transported without water and stored in a freezer in order to avoid loss of isopods from the mussels' mantle cavities.

After its shell length was determined, each mussel was carefully opened and the number and position of isopods recorded. Isopods from each mussel were kept in separate small glass tubes for further analysis of sex and length. The length of each isopod was measured using a binocular microscope with a calibrated ocular micrometer. The length of an isopod was defined as the distance between the frontal margin of the cephalon and the tip of the telson in stretched animals.

In addition, we determined the dry-tissue weight of each mussel (after 24 h at 100°C), to calculate regression lines representing the relationship between shell length and dry-tissue weight in mussels with and without isopods.

RESULTS

Occupation of mussels by isopods

In Gregorio and Santiago Bays, 18.3% and 7.6%, respectively, of the mussels investigated were occupied by the isopod *Edotea magellanica* (Fig. 1). The size class distribution of *Mytilus chilensis* from the natural banks in Gregorio Bay and Santiago Bay and the number of mussels occupied by isopods in different mussel size classes are presented in Figure 1. The most numerous size class of mussels with isopods was 40–45 mm in Gregorio Bay, and 30–35 mm in Santiago Bay.

In Gregorio Bay, mussels smaller in shell length than 25 mm were not occupied by isopods. In Santiago Bay, among the 38 mussels found in the 20–25 mm class, only one mussel was occupied by isopods. The absence of isopods in small mussels may be explained by the isopod's relatively large size: up to 13.4 mm in length in the mantle cavity of *Mytilus chilensis.* In neither of the bays investigated were isopods found in mussels larger than 50 mm in shell length. However, few specimens of these large size classes exist, so that the complete absence of isopods in these mussels could not be verified with certainty.

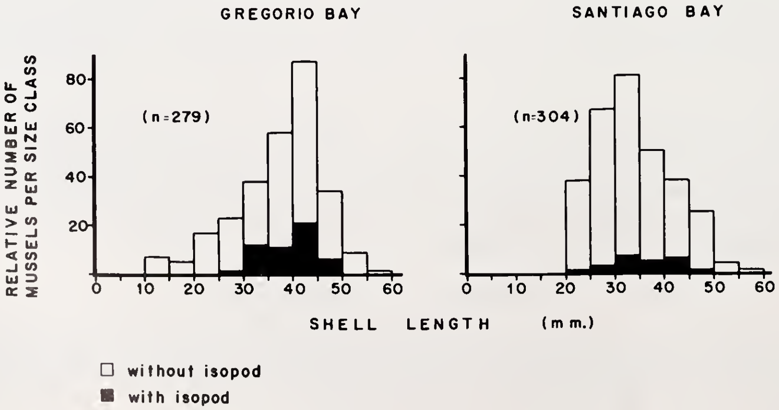


FIGURE 1. Size class distribution of *Mytilus chilensis* from the two different mussel banks investigated, and number of mussels occupied by isopods, by size classes.

Influence of the isopod on mussel meat content

To find whether isopod occupation influenced the meat content of *Mytilus chilensis*, the dry-tissue weights of the mussels with and without isopods were plotted against shell lengths (Fig. 2). Regression lines and covariance analysis (Sokal and Rohlf, 1969; Rohlf and Sokal, 1969) showed no such influence on meat content. Furthermore, from microscopic investigations, especially on the very delicate gills, it appears that specimens of *Edotea magellanica* do not damage the host's tissues.

Number, size, and sex-ratio analysis of isopods

Figures 3 and 4 show the number, size, and sex of each *Edotea magellanica* found in the mantle cavity of *Mytilus chilensis* from the two bays. An analysis of mussels occupied by isopods reveals that in Gregorio Bay and Santiago Bay, 29.4% and 65.2%, respectively, of the mussels contained only one isopod, a female. Two isopods, one female adult and one male adult, were found in 23.5% (Gregorio Bay) and 21.7% (Santiago Bay). Findings of two (7.8%) or three (2.0%) male adults (in addition to the female), which may function as progenitors alternately in one

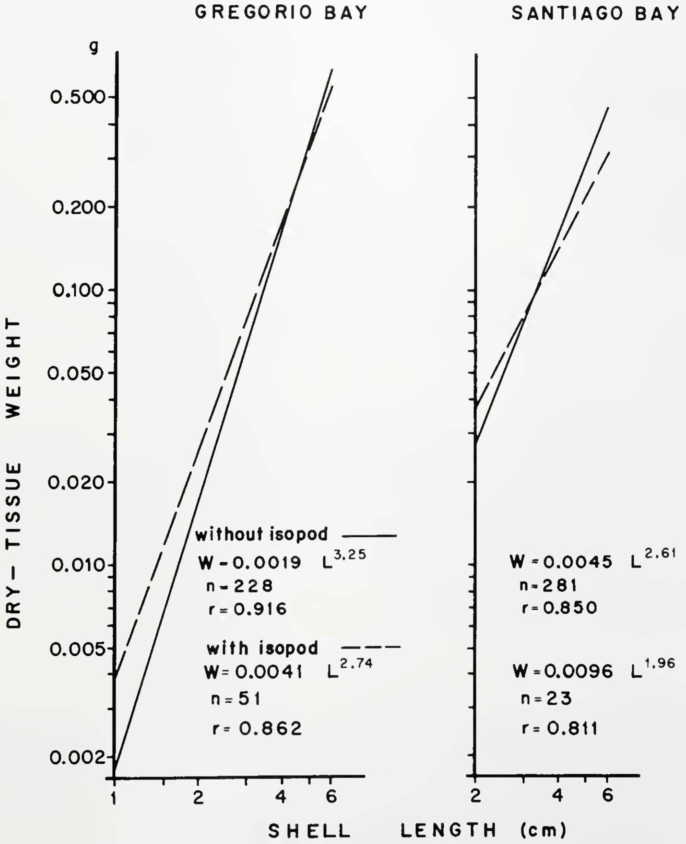


FIGURE 2. Correlation between shell length and dry-tissue weight in *Mytilus chilensis*, with and without isopods.

host (Fig. 3), were an exception in this isopod. In none of the mussels investigated was only one isopod, a male, found.

We found mussels occupied by up to 65 isopods. More than three males were found in 37.3% of the mussels occupied by isopods from Gregorio Bay and in 13.1% of those from Santiago Bay. However, these mussels always contained only one mature female, apparently the originator of all the generations in a given mussel. Specimens of a new isopod generation were differentiated in sex before leaving the host. The males reached their final length in the host where they were born (Figs. 3 and 4), while the females reached a maximum length of less than 5.5 mm before

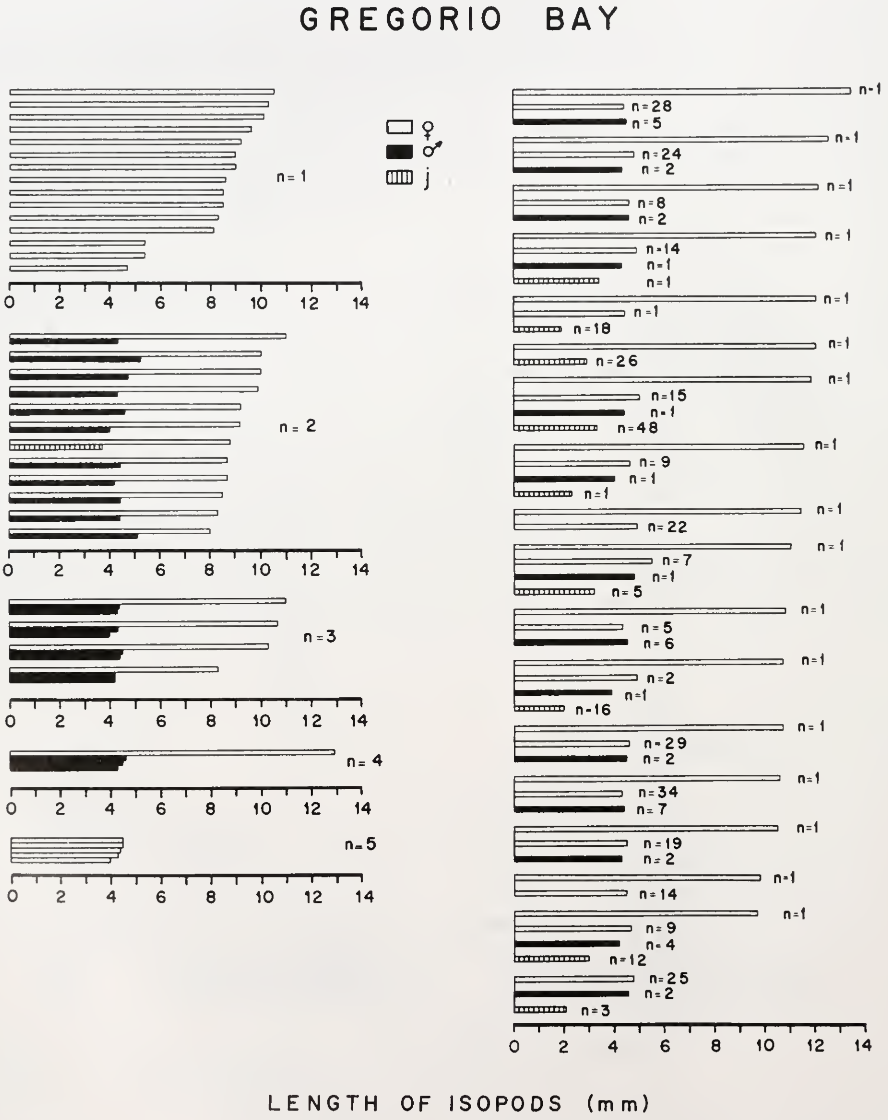


FIGURE 3. Number, size and sex of the isopods found in the mantle cavity of *Mytilus chilensis* in Gregorio Bay.

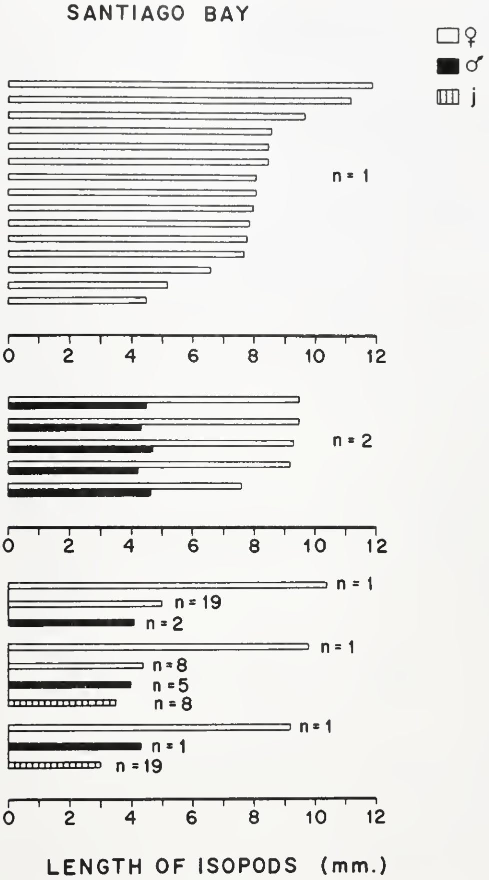


FIGURE 4. Number, size and sex of the isopods found in the mantle cavity of *Mytilus chilensis* in Santiago Bay.

leaving the host. Thus, in all cases where a mussel contained more than one female isopod, there were no females, the female progenitor excluded, with a length between 5.5 mm and 9.7 mm. The growth period that corresponds to this range of length seems to take place in newly occupied mussels (Figs. 3 and 4) or, if life is continued as a free-living isopod, in the corresponding environment. Furthermore, (Figs. 3, 4) the number of immature females always greatly exceeded that of males.

DISCUSSION

Presence of the isopod *E. magellanica* had no detectable negative effect on the meat content of *M. chilensis*. Therefore, we conclude that the isopods were not feeding on mussel food particles—that is, particles transported along the marginal food grooves of the gills and concentrated by the palps before entering the digestive tract. The isopod may be feeding on the pseudofeces, which are accumulated in the mantle cavity and which contain, entangled in mucus, energy-rich food particles.

Thus, *Edotea magellanica* should be considered a commensal, as defined by

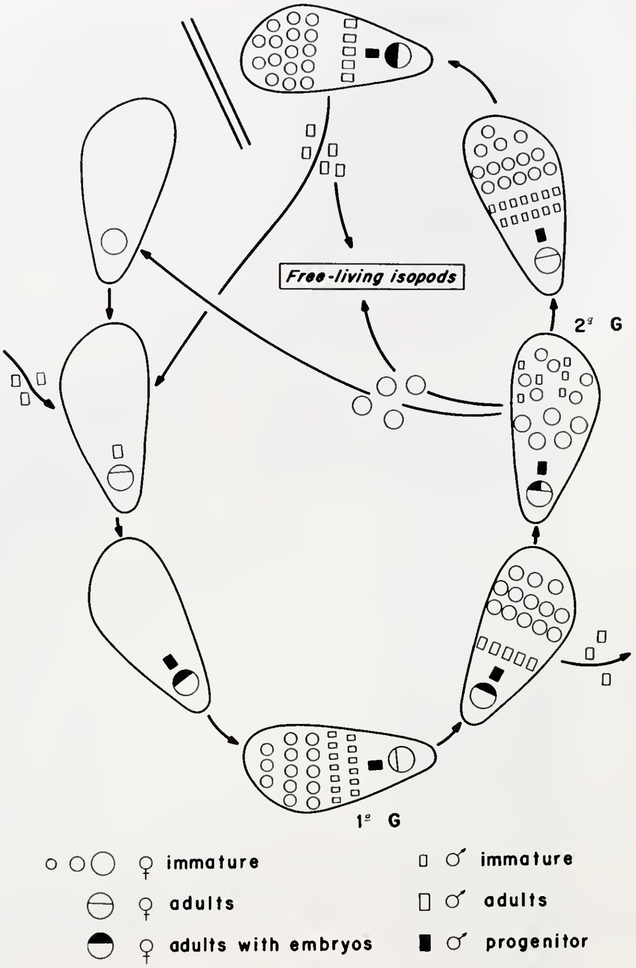


FIGURE 5. Schematic illustration of the life cycle of the isopod *Edotea magellanica* from the mantle cavity of *Mytilus chilensis*. Cycle from top left.

Cheng (1967, p. 6): "Commensalism describes that type of more or less intimate relationship during which the commensal generally derives physical shelter from the host, is nourished on foods that are associated but not a part of the host, and is not metabolically dependent on the host".

Edotea magellanica can also be found as a free-living isopod. As mentioned above, the species was found free-living when first described in 1871. On the northern side of the Straits of Magellan, this isopod was found burrowing in shell gravel and coarse sand under stones in the intertidal area. Thus, we consider *Edotea magellanica* a non-obligatory commensal. *Edotea magellanica*, as a commensal, is not restricted to a specific host. Specimens were found in other bivalves, such as *Mulinia sp.* Gray (estuary of Lingue River, 39° S, southern Chile; authors' unpublished observations) and *Aulacomya ater* (Molina) (information obtained by fishermen in Punta Arenas).

From the analysis of the data presented in Figures 3 and 4, the life cycle of

Edotea magellanica as non-obligatory commensal can be constructed as schematically illustrated in Figure 5.

From the large number of mussels (Gregorio Bay, 29.4%; Santiago Bay, 65.2%) occupied only by one isopod, and that a female, and the fact that no mussels were occupied by a lone male, we concluded that the mussel is occupied first by a female. The female isopod may then be joined by a male. A multiple "infection" of one mussel by more than one female adult seems to be avoided by a mechanism not yet understood, because in none of the mussels investigated did we find two or more adult (defined as >5.5 mm in length) females.

The new isopod generation develops in the marsupium and continues in the mantle cavity of the mussel when the embryos reach a length of about 1.9 mm (minimum length of isopods found free in the mantle cavity).

Since no mussel contained more than one female isopod longer than 5.5 mm, we conclude that all females of the new generation leave the mussel in which they were born.

The males may reach their final length in the mussel in which they were born, and leave their host before the emigration of the females of the same generation. This conclusion is supported by the fact that some mussels contain only young females of a generation under consideration, while a subsequent generation is growing up.

ACKNOWLEDGMENTS

We wish to thank Prof. Leonardo Guzman of the Hydrobiological Laboratory of the Instituto de la Patagonia (Punta Arenas) for his kindness in providing samples for this study. E. Jaramillo and J. Navarro would like to thank all the members of this institute for their generous arrangements with regard to laboratory space during their stay in Punta Arenas in August 1978.

LITERATURE CITED

CHENG, T. C. 1967. Marine molluscs as hosts for symbioses. Pp. 1–424 in F. S. Russell, Ed., *Advances in marine biology*, Vol. 5. Academic Press, London.

GALTSOFF, P. S. 1964. The American oyster. *Fish. Bull. U. S. Fish Wildl. Serv.* **64:** 1–480.

ROHLF, F. J., AND R. R. SOKAL. 1969. *Statistical tables.* W. H. Freeman and Co., San Francisco. 253 pp.

SCHULTZ, G. A. 1969. *The marine isopod crustaceans.* Wm. C. Brown Company Publishers, Dubuque, Iowa. 359 pp.

SINDERMANN, C. J. 1970. *The principal diseases of marine fish and shellfish.* Academic Press, New York and London. 369 pp.

SOKAL, R. R., AND F. J. ROHLF. 1969. *Biometry.* W. H. Freeman and Co., San Francisco. 776 pp.

Reference: *Biol. Bull.*, **160**: 114–122. (February, 1981)

CIRCADIAN FLUCTUATIONS IN TOTAL PROTEIN AND CARBOHYDRATE CONTENT IN THE SLUG *LAEVICAULIS ALTE* (FERUSSAC, 1821)

T. PAVAN KUMAR, R. RAMAMURTHI, AND K. SASIRA BABU[1]

Department of Zoology, S. V. University, Tirupati 517502, India

ABSTRACT

Rhythmic fluctuations in total protein and carbohydrates in the central nervous system, midgut gland, and foot muscle of *Laevicaulis alte* were not in phase with each other. Fluctuations were largest in the midgut gland. The occurrence of crest values during dark hours for total protein and light hours for total carbohydrates, high protein levels during the dark span and high carbohydrates during light, may be related to the animal's motor functions. Percent loss of water content was bimodal in all three tissues studied.

INTRODUCTION

In molluscs, free or combined amino acids form macromolecules, predominantly proteins (Florkin and Bricteux, 1972). Rhythmic fluctuations in various amino acids and proteins over 24-h periods were reported in vertebrates (see Sollberger, 1965) and in the abdominal ganglia of sea hares (Loh and Peterson, 1973). Strumwasser and Wilson (1976) could not detect a rhythm in the formation of protein fractions in the R15 neuron in the abdominal ganglia of *Aplysia*.

Blood sugar levels range widely both within and between species of molluscs, but are generally lower than those of vertebrates (Goddard and Martin, 1966). Depletion of glycogen during starvation in *Cryptozona semirugata* (Ramamurthi and Subramanyam, 1976) is consistent with glycogen use for energy (Meenakshi, 1956). Though there are several studies on cyclical phenomena in carbohydrates among vertebrates (see Sollberger, 1965) such studies on molluscs are scanty.

Physiological rhythms have been found in the acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and acetylcholine (ACh)-AChE systems in the slug *Laevicaulis alte* (Pavan Kumar and Sasira Babu, 1978; Pavan Kumar *et al.*, in press); in ACh, AChE, and electrical activity in the snail *Cryptozona ligulata* (Reddy *et al.*, 1978). The present investigation examines rhythmic fluctuations in carbohydrates and proteins, major sources of energy in the slug *Laevicaulis alte.*

MATERIALS AND METHODS

Adult, 6–10 gm slugs (*Laevicaulis alte*) were collected during September 1975 from fields around Tirupati (India) and acclimated to laboratory conditions for 10 days in large wooden boxes containing wet mud. Ambient temperature was maintained at 26 ± 2°C and relative humidity 85 ± 5% under a 12:12 (0600 to 1800:1800 to 0600 h) light:dark (L:D) regimen. Ambient temperature and relative

---

Received 23 July 1979, accepted 10 November 1980.

Abbreviations: CNS, central nervous system; L:D, light:dark ratio; φ, acrophase.

[1] Present address: Professor and Head, Department of Zoology, S.V.U.P.G. Extension Centre, Kavali 524201, India.

humidity in the fields where animals were collected were not determined. The animals were fed fresh leaves daily *ad libitum*. (Feeding always coincided with the nocturnal active phase of locomotor rhythm; Pavan Kumar and Sasira Babu, 1979.) Since the slugs are hermaphrodites, sex does not influence rhythmic patterns.

Isolation of tissues and experimental procedure

For experimentation, the 24-h day was divided into six 4-h periods beginning at 0800, 1200, 1600, 2000, 0000, and 0400 h. For 4 days during September 1975, central nervous system (CNS), midgut gland, and foot muscle taken from at least three animals at each period were pooled. The isolated tissues were cleared of body fluids and stored in prechilled glass tubes until analyzed.

Estimate of protein content

Total protein levels in the tissue homogenates were estimated following the method of Lowry *et al.* (1951). Protein content was expressed as micrograms/milligram wet weight of fresh tissue.

Estimate of carbohydrate content

Total carbohydrate levels in tissue homogenates were estimated following the method of Carroll *et al.* (1956).

Tissue homogenates of 1% (weight: volume) were made in 10% trichloroacetic acid (TCA) solution. To 0.5 ml of the centrifuged (3000 rpm for 15 min) clear supernatant, 5.0 ml of anthrone reagent was added and the combination boiled for 10 min in a water bath. The tubes, with their contents, then were immediately cooled. A standard sample containing a known quantity of analar glucose solution was always run along with the experimental samples. The color was measured at 620 nm in Bausch and Lomb Spectronic 20 against a reagent blank. The level of the content was expressed as milligrams/gram wet weight of fresh tissue.

Estimate of water content in the tissues

The CNS, midgut gland, and foot muscle from the slugs were isolated and collected into tinfoil planchets whose empty weights had been previously determined. After the tissues were weighed, they were placed at 100°C in a hot air oven. Weights were determined every 24 h until two readings coincided. Percent loss of water was calculated from the differences in the tissue weights.

Analysis of results

Total protein and carbohydrate content were analyzed for significant differences between data points in a cycle and significant differences between crests and troughs, using Student's t test.

The single cosinor method of Koukkari *et al.* (1974) also was applied to characterize rhythms. Amplitude values were calculated through trigonometric functions and their significance determined through F values obtained. Significance or lack of it at a given level (here $p = 0.05$) is determined based on whether the calculated F value is greater (significant) or less (not significant) than a corresponding value from the F table.

Since at 0000 h the protein content in the CNS exhibited no statistical signif-

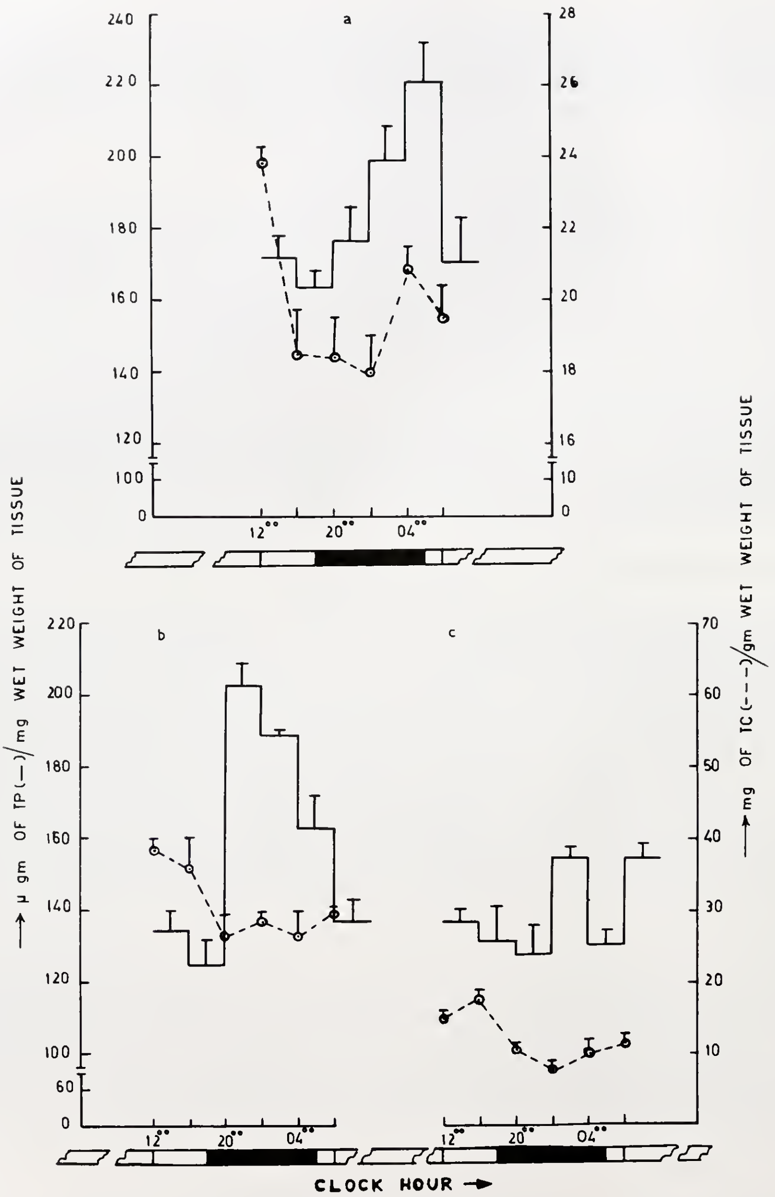


FIGURE 1. Cyclical fluctuations as a function of time in total protein (solid line) and carbohydrate (broken line) contents in the CNS (a, above), midgut gland (b, below left) and foot muscle (c, below right) of *Laevicaulis alte* at L:D 12:12 (0600 to 1800:1800 to 0600 h). Protein content expressed as μgm/mg wet weight of fresh tissue and carbohydrate content as mg/gm wet weight of fresh tissue. Vertical lines = standard error. CNS = central nervous system; MG = midgut gland; FM = foot muscle; Open block = light and bold block = dark hours of 24 h day; TC = total carbohydrate; TP = total protein.

icance for amplitude values, the next time period in the scale (0400 h, when the amplitude was significant) was selected to test amplitude.

The 24 h day was arbitrarily divided into two spans: 0800, 1200, and 1600 h,

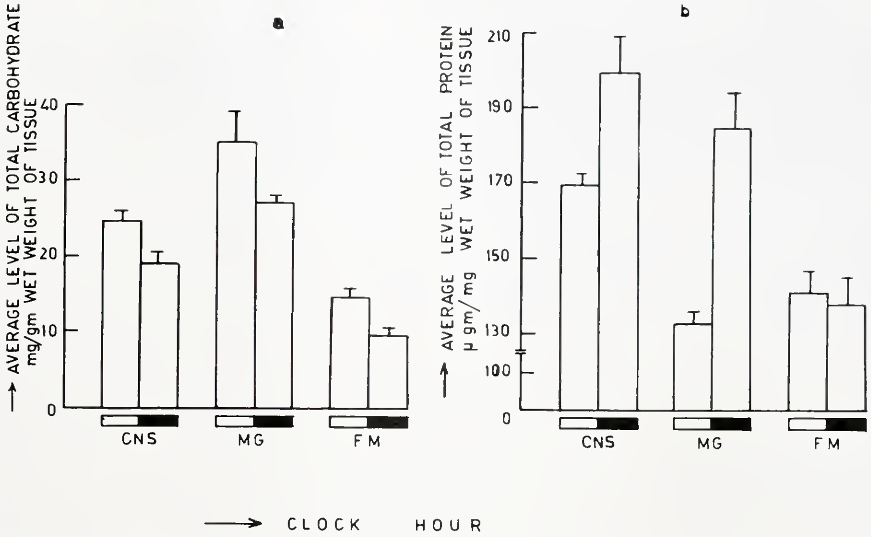


FIGURE 2. Average levels of total carbohydrate (a, left) and protein (b, right) in CNS, midgut gland, and foot muscle of *Laevicaulis alte* during light (0800 to 1600 h, open block) and dark (2000 to 0400 h, solid block) spans of 24 h solar day. CNS = central nervous system; MG = midgut gland; FM = foot muscle.

occurring during light hours (the light span) and 2000, 0000, and 0400 h, occurring during dark hours (the dark span). The averages during the two spans were plotted as histograms (Fig. 2). Percent differences between the average values for light and dark spans were calculated (Table II).

RESULTS

CNS protein and carbohydrate levels

Total protein and carbohydrate levels in the CNS crested at 0400 and 1200 h, respectively (Fig. 1a). Total protein was high during the dark span (Fig. 2b) and carbohydrates high during the light span (Fig. 2a).

Computed crests at –346° for total protein and –154° for total carbohydrate levels (Table I) show that the calculated crest values were closely synchronized with the visual crests in chronograms. (Converted to time, the computed crests were at 0304 h for total protein and at 1016 h for total carbohydrates). While the amplitude values in total carbohydrate content were not significant, they were significant for total protein content (Table I).

Protein and carbohydrate levels in midgut gland

Midgut gland values peaked at 1200 and 2000 h for carbohydrates and proteins respectively (Fig. 1b). As in the CNS, the average values were higher during light for carbohydrates (Fig. 2a) and dark for proteins (Fig. 2b). Computed acrophase (φ, lag from beginning time to the peak) values were synchronized with chronogram crests (Table I). Amplitude values were significant for both protein and carbohydrate (Table I).

TABLE I

Rhythm characteristics M (mesor), A (amplitude), significance, and φ (computed acrophase) for total protein and carbohydrate content in nervous system (CNS), midgut gland (MG) and foot muscle (FM) of Laevicaulis alte *under L:D 12:12 regimen. Amplitude values significant* ($P = 0.05$) *when* $F(2,3) > 9.6$. *Not significant when* $F(2,3)$ ($P = 0.05$) < 9.6. ** Reference local time 0400 h (otherwise 0000 h).*

Source of variables estimated	M ± SD	A ± SD	A Significant/ not significant (S, NS)	φ (range)
TOTAL PROTEIN				
CNS	184.27 ± 19.97	26.71 ± 6.02	S (12.18)	−346°* (−319.5° to 348.5°)
MG	158.04 ± 28.97	41.56 ± 9.08	S (17.88)	−1° (−353° to −9.5°)
FM	139.87 ± 11.38	4.5 ± 2.5	NS (0.13)	−67.5° (−52.5° to −83°)
TOTAL CARBOHYDRATES				
CNS	19.86 ± 2.04	2.09 ± 0.08	NS (1.64)	−154° (−138° to −177°)
MG	31.01 ± 4.67	6.22 ± 2.6	S (11.79)	−203.5° (−184.5° to −230°)
FM	12.08 ± 3.19	4.32 ± 2.5	S (14.96)	−206.5° (−177.5° to −223°)

Protein and carbohydrate levels in foot muscle

In foot muscle, total protein content in the chronogram crested at 0000 and 0800 h (Fig. 1c). But the calculated crest occurred at 0430 h ($\varphi = -67.5°$) (Table I). As in midgut gland, the crest in total carbohydrate content in foot muscle at 1600 h (Fig. 1c) is comparable to a calculated crest at 1346 h ($\varphi = -206.5°$) (Table I).

Protein content in foot muscle did not fluctuate significantly from light to dark spans and carbohydrate levels were large during light (Fig. 2).

Water content in CNS, midgut gland, and foot muscle

In all the tissues, percent loss of water content was bimodal. CNS exhibited the maximum loss and midgut gland the least (Fig. 3).

Other findings

The differences between maximum and minimum values of physiological variables were statistically highly significant at $P < 0.001$ for total carbohydrates in CNS and foot muscle and for total protein in CNS and midgut gland. Differences between high and low values of total carbohydrates in midgut gland and total protein in foot muscle were significant at $P < 0.01$. On overview, carbohydrates, with crests (Figs. 1a to 1c) and higher average values during the light span (Fig. 2), and proteins with the opposite pattern, exhibited uniformity for all the tissues despite discrepancies in the "times" of the crests. Midgut gland registered the

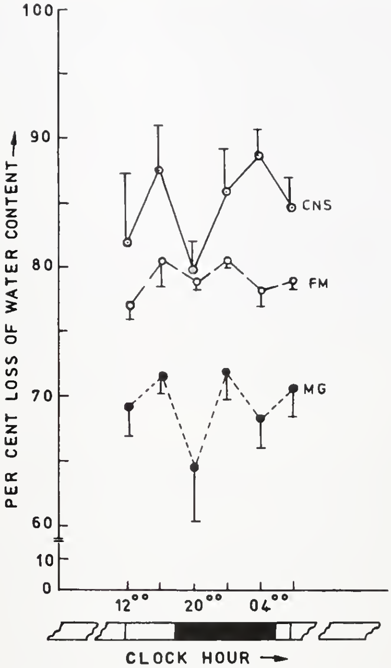


FIGURE 3. Percent loss of water content in CNS, midgut gland, and foot muscle of *Laevicaulis alte* during 24 h L:D regimen of solar day. Vertical lines = standard error. CNS = central nervous system; MG = midgut gland; FM = foot muscle.

highest amplitude for both the variables (Table I). When percent gain and loss from light to dark span and *vice versa* were compared for the variables in the tissues, total protein content increased from light to dark span and an opposite trend was observed for total carbohydrate (Table II; Fig. 2).

DISCUSSION

Laevicaulis alte exhibits a monophasic pattern of motor rhythm, with its active phase during the dark hours of a 24 h day (Pavan Kumar and Sasira Babu, 1979).

TABLE II

Comparison of percent differences in the physiological variables in central nervous system (CNS), midgut gland (MG) and foot muscle (FM) of Laevicaulis alte *under L:D 12:12. + = increment; − = decrement.*

Source of the physiological variable	Light to dark span	Dark to light span
TOTAL PROTEIN		
CNS	+17.95	−15.22
MG	+38.54	−27.82
FM	−2.19	+2.24
TOTAL CARBOHYDRATE		
CNS	−6.64	+7.11
MG	−21.73	+27.75
FM	−34.85	+53.48

This is coupled to cyclical fluctuations in physiological variables such as heartbeat (Pavan Kumar and Sasira Babu, 1976), AChE and BuChE levels (Pavan Kumar and Sasira Babu, 1978), and ACh-AChE system (Pavan Kumar *et al.*, in press), which all crest during the active phase. Similar phenomena were observed in snails (Reddy *et al.*, 1978) and calotes and mice (Pavan Kumar *et al.*, 1979). In the present investigation, an inverse relationship was observed in fluctuations of total protein and carbohydrate levels. Similar inverse relationships have been found in the rates of heartbeat and pause in the slug (Pavan Kumar and Sasira Babu, 1976), 5-hydroxytryptamine content in different parts of the brain of turtles (Quay, 1967), and glycogen content in mice (Barnum *et al.*, 1958). The opposition of total protein to the rhythmic pattern of total carbohydrate content suggests that carbohydrates accumulate during the rest period. Accumulation from dietary sources would account for higher levels of carbohydrates during the light span, since the active phase of motor rhythm and the feeding of the animal coincide (Pavan Kumar and Sasira Babu, 1979).

The amplitude values (Table I) for protein and carbohydrate in the midgut gland clearly document that the fluctuations, and thus the turnover of the constituents, are larger in this gland than in other tissues examined. The fluctuations of the constituents in this organ clearly are statistically significant. Acceptance of the null hypothesis for total carbohydrates in CNS and total protein in foot muscle, reflecting no significant amplitude values, suggests a different situation. In the CNS carbohydrates probably are used for energy while in foot muscle proteins may meet requirements for other associated reactions. This assumption is supported by the small differences in percent loss and gain for total carbohydrates in CNS and total proteins in foot muscle (Table II).

The cresting and high average levels in total protein content during dark hours suggest that use or turnover of protein (and possibly associated reactions) may be high during the active phase of motor activity in *Laevicaulis.* Protein use (Meenakshi, 1956) is associated with higher levels of certain enzymes in aestivating *Pila globosa* (Murthy *et al.*, 1974). The transamination reactions in slugs and cockroaches (Pavan Kumar, 1976; Pavan Kumar and Sasira Babu, in press; Sasira Babu *et al.*, 1977; Vijayalakshmi *et al.*, 1978) are high during the animals' active phases of motor rhythm.

The cresting and high average levels of the total protein content during dark periods in all three tissues suggests that protein synthesis might be high during the inactive phase, since accumulation requires time. Protein formation might be high during active phase. Loh and Peterson (1973) observed that the 12K protein fraction in the abdominal ganglia of *Aplysia* is high at dawn. This is in association with the circadian motor pattern (Jacklet, 1972). However, Strumwasser and Wilson (1976) could not detect a circadian pattern in formation of protein in the abdominal ganglion of *Aplysia.* It also has been suggested that the time of dissection has a bearing on the physiological processes of the ganglion (Audesirk and Strumwasser, 1975).

Circadian rhythms in carbohydrates in different groups of animals have been reviewed (see Sollberger, 1965). Among invertebrates, circadian rhythms occur in carbohydrates in crustaceans (Dean and Vernberg, 1965) and insects (Nowosielski and Patton, 1964). Relationship of carbohydrates to energy source in *Laevicaulis alte* is reflected in krebs cycle enzymes (succinate dehydrogenase, Pavan Kumar, 1976; Pavan Kumar and Sasira Babu, in press a) and associated enzymes like aspartate and alanine aminotransferases (Pavan Kumar, 1976; Pavan Kumar and Sasira Babu, in press b). Thus, high total carbohydrate levels during the inactive phase might result from the need to meet energy requirements for synthesis of various biological constituents while decreased levels during the active phase reflect utilization of carbohydrates for motor functions that demand energy. In conformity with this, the observed fluctuations in total carbohydrate content were greatest in foot muscle (Table II), which is actively engaged in locomotion.

ACKNOWLEDGMENTS

The authors thank Professor K. S. Swami, Professor and Head, Department of Zoology, S. V. University, Tirupati, for facilities. Pavan Kumar thanks CSIR (India) for the award of a postdoctoral fellowship. The help of Mr. N. Sivarami Reddy, Department of Zoology, S. V. University, Tirupati, is acknowledged.

LITERATURE CITED

AUDESIRK, T. J., AND F. STRUMWASSER. 1975. The circadian rhythm of the neuron R15 of *Aplysia californica: In vivo* photoentrainment. *Proc. Nat. Acad. Sci. USA* **72:** 2408–2412.

BARNUM, C. P., C. D. JARDETZKY, AND F. HALBERG. 1958. Time relations among metabolic and morphologic 24-hour changes in mouse liver. *Am. J. Physiol.* **195:** 301–310.

CARROLL, N. V., R. W. LONGLEY, AND J. H. ROC. 1956. Glycogen determination in liver and muscle by use of anthrone reagent. *J. Biol. Chem.* **22:** 583–593.

DEAN, J. M., AND F. J. VERNBERG. 1965. Variations in blood glucose level of crustacea. *Comp. Biochem. Physiol.* **14:** 29–34.

FLORKIN, M. J., AND G. BRICKTEUX. 1972. Nitrogen metabolism in molluscs. In M. Florkin and B. T. Scheer, Eds., *Chemical Zoology, Vol. VII. Mollusca*, Academic Press, New York and London.

GODDARD, C. K., AND W. A. MARTIN. 1966. Carbohydrate metabolism. Pp. 275–308 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of Mollusca, Vol. II.* Academic Press, New York and London.

JACKLET, J. W. 1972. Circadian locomotor activity in *Aplysia. J. Comp. Physiol.* **79:** 325–341.

KOUKKARI, L. W., H. D. STANELY, F. HALBERG, AND J-K. LEE. 1974. Circadian leaflet movements; student exercise in chronobiology. *Chronobiologia* **1:** 281–302.

LOH, Y. P., AND R. P. PETERSON. 1973. The circadian rhythm of protein synthesis in a molluscan neuron R15 of *Aplysia. Soc. Neurosci.* **3:** 137.

LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265.

MEENAKSHI, V. R. 1956. Seasonal variations in the glycogen and fat content in the apple snail, *Pila virens* (Lamarck). *J. Zool. Soc. India* **8:** 57–62.

MURTHY, CH. R. K., T. PAVAN KUMAR, K. SASIRA BABU, AND K. S. SWAMI. 1974. On the possible significance of enhanced glutamate dehydrogenase activity in aestivated *Pila globosa. Veliger* **16:** 419–422.

NOWOSIELSKI, J. W., AND R. L. PATTON. 1964. Daily fluctuations in the blood sugar concentration of the house cricket, *Gryllus domesticus* L. *Science* **144:** 180–181.

PAVAN KUMAR, T. 1976. Locomotor and physiological rhythms in the slug, *Laevicaulis alte* (Ferussac, 1821). *Ph.D. Dissertation*, S. V. University, Tirupati, India, Library accession No. 191158.

PAVAN KUMAR, T., AND K. SASIRA BABU. 1976. Circadian rhythmicity in the heartbeat of the slug, *Laevicaulis alte* (Ferussac, 1821). *Ind. J. Expt. Biol.* **14:** 364–365.

PAVAN KUMAR, T., AND K. SASIRA BABU. 1978. Diurnal variations in cholinesterase activities in the slug, *Laevicaulis alte. Experientia* **34:** 612–613.

PAVAN KUMAR, T., AND K. SASIRA BABU. 1979. Locomotor rhythms in the slug, *Laevicaulis alte* (Ferussac, 1821). *Ind. J. Expt. Biol.* **17:** 421–423.

PAVAN KUMAR, T., G. R. REDDY, S. VIJAYALAKSHMI, AND K. SASIRA BABU. 1979. Cyclical acetylcholinesterase activity in diurnal *Calotes nemoricola* and nocturnal *Mus booduga. J. Interdiscipl. Cyc. Res.* **10:** 139–144.

QUAY, W. B. 1967. Twenty-four-hour rhythms in cerebral and brainstem contents of 5-hydroxytryptamine in a turtle, *Pseudemys scripta elegans. Comp. Biochem. Physiol.* **20:** 217–221.

RAMAMURTHI, R., AND O. V. SUBRAMANYAM. 1976. Organic composition of haemolymph, hepatopancreas and foot muscle of the garden snail *Cryptozona semirugata* (Beck) with reference to starvation. *Ind. J. Expt. Biol.* **14:** 492–493.

Reddy, G. R., T. Pavan Kumar, P. Murali Mohan, and K. Sasira Babu. 1978. Diurnal variations in physiological activities in the garden snail *Cryptozona ligulata*. *J. Comp. Physiol.* **125:** 59–66.

Sasira Babu, K., T. Pavan Kumar, and S. Vijayalakshmi. 1977. On circadian rhythmicity of aminotransferases in cockroach and slug. *XV Int. Ethol. Conf.*, Bielefeld.

Sollberger, A. 1965. *Biological rhythm research*. Elsevier Publishing Company, Amsterdam-London. 461 pp.

Strumwasser, F., and D. L. Wilson. 1976. Patterns of proteins synthesized in the R15 neuron of *Aplysia*. *J. Gen. Physiol.* **67:** 691–702.

Vijayalakshmi, S., T. Pavan Kumar, and K. Sasira Babu. 1978. Rhythmicity of aminotransferases in the cockroach, *Periplaneta americana*. *Experientia* **34:** 192–193.

Reference: *Biol. Bull.*, **160:** 123–135. (February, 1981)

APOMICTIC PARTHENOGENESIS IN A HERMAPHRODITIC TERRESTRIAL SLUG, *DEROCERAS LAEVE* (MÜLLER)

NANCY L. NICKLAS[1] AND RICHARD J. HOFFMANN[2]*

[1]*Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260; and*
[2]*Department of Zoology, Iowa State University, Ames, Iowa 50011.*

ABSTRACT

Electrophoretic studies and breeding experiments show that the terrestrial pulmonate slug, *Deroceras laeve*, usually reproduces by apomictic parthenogenesis, although reciprocal outcrossing in complete hermaphrodites occurs occasionally. The sexual status of the species is controlled by the environment, with low temperatures and/or exposure to light inhibiting the development of male organs. Both hermaphrodites and females reproduce parthenogenetically. Sampling of an artificially established field population suggests that apomixis is also the rule in nature, and surveys of natural populations reveal that most populations consist of only a single clone, based on genetic identity at 20 enzyme loci. In spite of apomictic reproduction there is little heterozygosity in nature, although there is some allelic variation between populations.

INTRODUCTION

In recent years there has been renewed focus on the evolutionary implications of sexual and asexual reproduction (*e.g.*, Williams, 1975; Maynard Smith, 1978). Exploitation of both ameiotic asexual reproduction and sexual outcrossing in the same species can enhance a species' fitness (Marshall and Weir, 1979). Asexual reproduction replicates an entire genotype adapted to existing environmental conditions, favoring the expansion of a clone to the ecological limits imposed upon it. In contrast, outcrossing usually ensures genetic diversity among the offspring. In changing and unpredictable environments, sexuality provides release from genetic homogeneity, making available to selection new genetic combinations among the progeny and thereby avoiding potentially catastrophic extinction of a population by clonal elimination (*e.g.*, Shick *et al.*, 1979). A species with both modes of reproduction thus might meet all evolutionary contingencies by preserving coadapted blocks of genes in static or predictable conditions by asexual means, while allowing progressive genetic change through sexual recombination (Marshall and Weir, 1979).

Diverse modes of reproduction within hermaphroditic terrestrial slugs of the genus *Deroceras* Rafinesque (=*Agriolimax*) provide natural systems for experimental analysis of theoretical aspects of the evolutionary balance between asexual and sexual reproduction. Three species, *D. laeve* (Müller), *D. agreste* (Linnaeus), and *D. meridionale* (Reygrobellet), are capable of reproduction without outcrossing, a feature previously attributed to self-fertilization, based upon hermaphroditic morphology (Maury and Reygrobellet, 1964). These species show considerable

---

Received 14 July 1980, accepted 2 October 1980.

Abbreviations: CAN, TIM, BRI, SOA, PAS, MAR, MIS, HSS, BAY, MET—collection site codes for natural populations; EST1—esterase locus 1; EST2—esterase locus 2; IDH—isocitrate dehydrogenase; ME—malic enzyme; α-GPDH—α-glycerophosphate dehydrogenase.

* Author to whom reprint requests should be sent.

variation in penis morphology, with *D. laeve* sometimes entirely aphallic (Pilsbry, 1948; Reygrobellet, 1963). On the other hand, *D. reticulatum* (Müller) is an obligate outcrosser (Runham and Hunter, 1970; McCracken and Selander, 1980, and unpublished observations in our laboratory) in which hermaphroditic function is well characterized (Runham and Laryea, 1968).

In this paper we present evidence that one of these species, *Deroceras laeve*, takes advantage both of ameiotic asexual reproduction through apomictic parthenogenesis and of sexual outcrossing. Our explorations of laboratory and field populations of *D. laeve* with respect to mode of inheritance, fertility, fecundity, levels of enzyme polymorphism, and effects of rearing conditions upon sexual status suggest that this species exploits some, but perhaps not all, of the advantages available to it through the use of both modes of reproduction.

Materials and Methods

Collection and Rearing

Specimens of *Deroceras laeve* were collected from eight sites in and around Pittsburgh, Pennsylvania, and two in Canton, Ohio, by manual search of leaf litter and refuges such as stones and logs. Each site was given a three letter code; populations will be referred to by these codes. CAN and TIM are in Canton, Ohio; BRI is near Powdermill Nature Reserve, near Pittsburgh, Pennsylvania; SOA and PAS, in the vicinity of Ligoneer, PA; MAR from South Park, near Pittsburgh; MIS, HSS, BAY and MET in Mt. Lebanon, Pennsylvania.

Slugs were maintained in the laboratory on a modified slug diet (A. Gelperin, Princeton Univ., pers. comm.): a mixture of ground Purina Lab Chow, calcium carbonate, and vitamins (Vionate Vitamin-Mineral Powder for Pets, E. R. Squibb and Sons). Mass cultures of about 50 slugs were kept in plastic boxes lined with damp paper towels, with crumpled towels as refuges and oviposition sites. Clutches of eggs and small individuals were kept in 5.0 × 1.5 cm plastic Petri dishes; larger individuals were kept in small jars. Containers were changed every second day. Slugs were kept in darkness in drawers or in rooms with controlled photoperiods at either room temperature (22° ± 2°C) or 15° ± 2°C. Details of rearing conditions are given below as appropriate to the experiment.

Sexual Classification

To assess sexual status, we dissected slugs under a dissecting microscope. Each slug was classified as one of three morphological types: complete hermaphrodite, with well developed penis, prostate gland, spermathecum, and common duct; female, with penis and associated retractor muscles absent and prostate gland reduced or absent; or androgynous female, with penis reduced to a small bulge or nubbin. Pilsbry (1948) contains photographs of these variations in genitalia of *Deroceras laeve*.

Electrophoresis

Horizontal starch gels (13% W/V, Sigma) were cast in Plexiglas molds (0.8 × 12 × 20 cm) with covers and slot formers making wells of about 25 μl. Slugs were weighed and homogenized in a four-fold (W/V) volume of 0.05 *M* Tris-HCl, pH 7.1. Cutting small pieces from the posterior of large slugs did not affect the slugs' survival. Homogenates were centrifuged at 2600 × *g* for 20 min in a refrigerated Sorvall RC-5B centrifuge. After electrophoresis at 30 mA per gel for 4–5 h, gels were sliced and stained according to procedures modified from Brewer (1970), Selander *et al.* (1971), and Shaw and Prasad (1970). Twenty loci from 17 enzyme systems were scored using three buffer systems (Table I).

Genetic and morphological analysis of progeny

Initial experiments rearing slugs in complete isolation from egg to adult demonstrated that *D. laeve* could reproduce without outcrossing. To establish whether reproduction in isolation is due to selfing (or some other process involving recombination) or to apomictic parthenogenesis, we performed a series of breeding experiments using esterase loci (EST1 and EST2) as markers. Heterozygous strains were constructed for this purpose, since segregation cannot be detected in homozygotes without cytogenetic analysis. Ameiotic parthenogenesis is indicated if all offspring of an isolated heterozygote are likewise heterozygous. Meiosis is indicated by segregation of homozygotes and heterozygotes among progeny from such a parent.

Initially slugs from three natural populations (site codes BRI, PAS, SOA) were used to confirm reproduction without mating. Their offspring were reared in isolation from eggs to egg-laying adulthood. Pair matings were then made between homozygotes for different esterase alleles, rearing slugs in pairs from hatching to

TABLE I

Buffer systems used in electrophoresis of enzymes in Deroceras laeve. *Under each system are listed those enzymes with the number of loci for which acceptable resolution was obtained.*

Buffer system	# Loci
I. 0.1 M Tris-0.031 M Citrate, pH 7 Diluted 1:10 for gel. (Nichols and Ruddle, 1973)	
Hydroxybutyrate dehydrogenase (HBDH)	1
Isocitrate dehydrogenase (IDH)	1*
Malate dehydrogenase (MDH)	2
6-Phosphogluconate dehydrogenase (6PGDH)	1
Leucine aminopeptidase (LAP)	1
Malic enzyme (ME)	1*
Phosphoglucomutase (PGM)	1
II. Discontinuous Lithium Hydroxide (Selander *et al.*, 1971)	
β-naphthyl esterase (EST)	3*
αGlycerophosphate dehydrogenase (αGPDH)	1*
Aldehyde oxidase (AO)	1
"Tetrazolium oxidase" (TO)	1
III. Discontinuous tris citrate (Poulik) (Selander *et al.*, 1971)	
Glucose-6-phosphate dehydrogenase (G6PDH)	1
Xanthine dehydrogenase (XDH)	1
Glutamate oxalacetate transaminase (GOT)	1
Glutamate pyruvate transaminase (GPT)	1
Fructokinase (FK)	1
Phosphoglucoisomerase (PGI)	1

* Variable loci. All three esterase loci are variable.

adulthood. Three matings were made in duplicate involving the BRI, PAS, and SOA genotypes. The crosses were constructed so that progeny resulting from outcrossing were expected to be heterozygotes for either EST1, EST2, or both. EST1 heterozygotes are three-banded; EST2 heterozygotes are two-banded. BRI × SOA were expected to produce heterozygotes for EST2; PAS × SOA were expected to produce heterozygotes for EST1; while BRI × PAS were expected to produce heterozygotes at both esterase loci. One member of the second BRI × SOA pair died; the other was discarded, leaving five pairs in the experiment. Mated slugs were separated after several clutches had been laid and were allowed to continue to lay eggs for an additional 4 weeks. At that time parents were dissected and genotyped with samples of their offspring.

One large egg clutch was selected from each of the five matings. From each clutch 10 slugs, all of which proved heterozygous at the expected loci, were reared singly from hatching. Remaining clutchmates (also heterozygous) were reared in groups of five to fifteen, depending upon original clutch size. Except for one clutch, all of these slugs were reared from hatching on a controlled photoperiod of 12 h light: 12 h dark (LD 12:12) at 22°C. An exceptional clutch (BRI × SOA) was laid and thus hatched 5 days before parallel clutches from other matings. These animals were kept in darkness (LD 0:24, 22°C) until other clutches hatched, at which time all were moved to LD 12:12 at 22°C. Eggs were collected from isolated slugs and from siblings reared in groups. Each parent was dissected and genotyped electrophoretically with a sample of 15 of its newly hatched offspring.

Effects of rearing conditions on penis morphology

Deroceras laeve was reared from newly laid eggs (<24 h old) to adulthood under various combinations of pre- and post-hatch conditions to measure environmental effects on development of male organs. Four regimens were used: LD 0:24 at 22°C; LD 0:24 at 15°C; LD 12:12 at 22°C; LD 24:0 at 15°C. At hatching some groups of slugs from the four pre-hatching conditions were moved to the other regimens, so development continued under different conditions. This procedure allowed some discrimination of the sensitive stages of the life cycle. These 12 groups were supplemented with four in which slugs were reared from egg to adulthood under each of the four constant regimens. Each of the 16 experimental groups consisted of 25 slugs derived from the BRI × SOA strain and 25 from BRI × PAS. These genotypes, constructed as described above, were reared separately to assess genotype effects on morphology.

Adult slugs ($\geq$0.1 g) were dissected and scored for sexual condition. Gonads and spermatheca from five individuals of each genotype in each of the 16 groups were stained with aceto-orcein using the method of Humason (1972), squashed, and examined under a compound microscope for gametes.

Sexual status and structure of an artificial field population

A population of *Deroceras laeve* was established in the field from laboratory stocks in September 1978 at a site previously free of slugs. One hundred homozygous newly hatched slugs were released, half with an electrophoretically fast allele for isocitrate dehydrogenase (IDH) and half with a slower allele. The population was subsequently sampled at 4 week intervals from the next April (1979) through November by collecting all adults found by manual search in 2 h. Slugs of all samples were dissected for penis morphology. All samples but November's were genotyped for IDH to assess frequency of outcrossing. In addition, collections of at least 30 slugs were dissected in April, June, and August 1980. Gonads and spermatheca were searched for sperm or evidence of spermatogenesis by the above method.

RESULTS

Fertility and fecundity in the laboratory

Embryogenesis requires about 14 days at room temperature (22° ± 2°C). Hatching of eggs is synchronous, typically occurring within 1–2 h for a given clutch. *Deroceras laeve* begins to lay eggs 4 weeks after hatching, at a body weight of 0.06–0.1 g. Minimum generation time for this species is thus 6 weeks. These slugs continue to lay eggs at 2–3 day intervals as long as they live, which usually is 8 weeks post-hatching but can be as long as 13 weeks. Animals continue to increase in size throughout their lives; maximum size of a laboratory reared *D. laeve* was 0.51 g, compared to 0.43 g as a maximum that we found in nature. Clutch weight and size are proportional to the parents' body weight. Mean clutch size was 18 ± 8.5 (SE) eggs. Individuals laid as many as 350 eggs over their life spans.

Fertility in *Deroceras laeve* is usually above 98%, either in terms of the number of slugs producing eggs or in the number of eggs developing per clutch. Occasional clutches are completely infertile, especially small slugs' first clutches. Fertile adults collected from natural populations throughout the warm season readily laid eggs under laboratory conditions. Field populations consist of individuals of all sizes, reflecting continuous reproduction.

Rarely, in clutches of large eggs, multiple embryos must have developed in one egg, since the number of hatched slugs exceeded the number of eggs in the clutch. The number of embryos per egg is not known.

Apomictic parthenogenesis in Deroceras laeve

Deroceras laeve reproduces both sexually by outcrossing and by apomictic asexual means. Pair matings between the homozygotes for different esterase alleles were successful and reciprocal. Both members of each pair laid fertile eggs and these progeny were all heterozygous, producing the expected mutiple banded phenotypes. Apomictic parthenogenesis was confirmed in the next generation. Of 50 isolated slugs, 43 laid fertile eggs. From these, 23 parent-offspring comparisons were made. Each of the 15 tested offspring of all unmated heterozygotes were themselves heterozygous. This complete lack of segregation is consistent only with ameiotic parthenogenesis and not with self-fertilization. The binomial probability of obtaining this result in any family of 15 segregating progeny is 3×10^{-5}. We obtained this same result in 23 families. To our surprise, all sibs reared in groups were also parthenogenetic with one exception discussed below.

Parthenogenesis is also supported by gross internal morphology. We dissected 40 slugs reared in isolation and 60 reared in groups from hatching under LD 12:12 at 22°C. These animals were entirely aphallic, yet all isolated slugs reproduced, making self-fertilization highly improbable. The female reproductive system was complete, including a spermathecum. We did not search the reproductive tract for sperm. Mated slugs, reared under LD 0:24 at 22°C, were all hermaphrodites.

The exceptional clutch (above) differed with respect to both reproductive success and internal morphology. This clutch, produced by the mating BRI × SOA, was kept under LD 0:24, 22°C for 5 days after hatching, then transferred to LD

12:12, as described in Methods. Only three of the ten isolated slugs from this clutch laid eggs, and they laid 2 weeks later than parallel isolates from other clutches. Progeny from these isolates were uniformly heterozygous and hence produced parthenogenetically. The remaining 14 slugs from the exceptional clutch, reared together, were also slow to produce eggs. Seven individual egg clutches from this mass clutch consisted entirely of nonsegregating progeny (heterozygotes). The eighth clutch of 18 eggs gave rise to three electrophoretically fast homozygotes, ten heterozygotes, and five slow homozygotes. This result is not significantly different from the expected 1:2:1 ratio from a mating between heterozygotes ($P > 0.5$). We think it unlikely that this clutch was produced by self-fertilization since no sibling in isolation (indeed no slug in this study) selfed. We did not determine which among the 14 were laying eggs, although the segregating progeny was probably produced by only one, since all came from the same egg clutch.

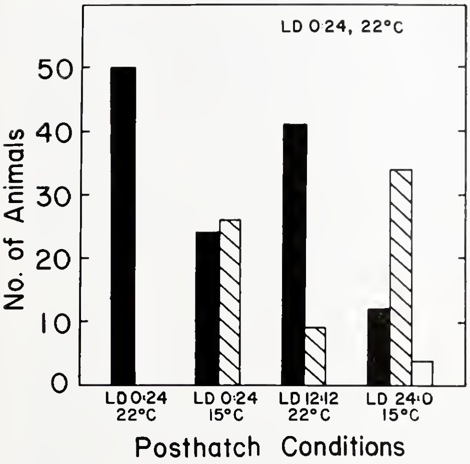
Of the 24 members of the initial exceptional clutch with delayed oviposition, 20 were hermaphrodites, including all 10 slugs reared in isolation. Four were female. Parthenogenesis thus occurs in hermaphrodites, as well as in females, although parthenogenetic reproduction seems to be delayed in hermaphrodites. To our knowledge, only hermaphrodites mate.

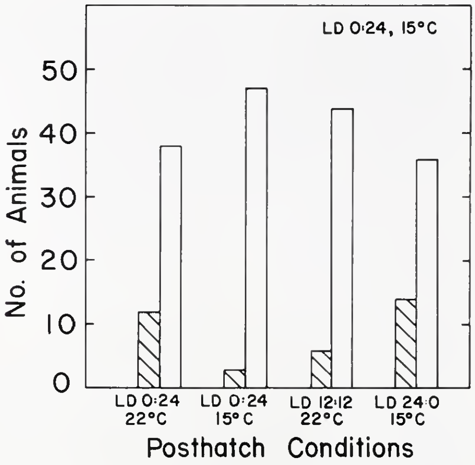
Effects of rearing conditions on penis morphology

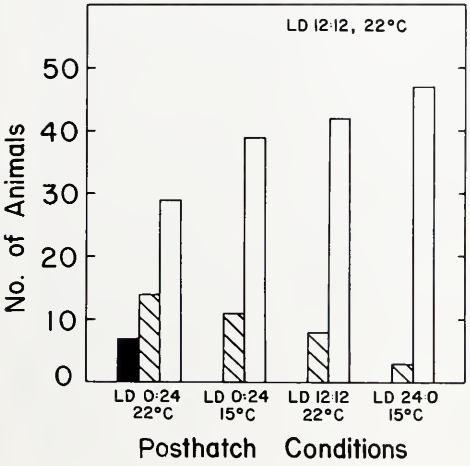
Differences in penis morphology among lab reared *Deroceras laeve* during breeding experiments suggested that exploration of effects of photoperiod and temperature on development of male end organs might be profitable. Asexually produced slugs of two hybrid strains, BRI × SOA and BRI × PAS, were used. With one exception, there were no significant differences in resulting phenotype frequencies between the two genotypes ($P > 0.05$, G test; Sokal and Rohlf, 1969). The exception involved slugs with embryonic development under LD 0:24 at 22°C and post-hatching conditions of LD 0:24 at 15°C. Twenty BRI × SOA hybrids became hermaphrodites; five were androgynous females. Only four of 25 BRI × PAS slugs were hermaphroditic; the remainder showed incomplete development of the penis (androgynous) ($P < 0.001$). No females were found in either group. Since this is the only exception and because we do not understand its basis, we pooled all data from the two genotypes.

Figure 1 summarizes the results of experiments where eggs developed under LD 0:24 at 22°C. Slugs reared from egg to adulthood under those conditions became hermaphrodites. The frequency of complete development of male organs was decreased by more than half by moving slugs to 15°C at hatching, regardless of photoperiod. These slugs tended to become androgynous females; only four females were found in the post-hatching group LD 24:0 at 15°C. Cooler temperatures, regardless of photoperiod, seem to inhibit completion of male organs. The frequency of hermaphrodites was also decreased by exposing slugs to 12 or 24 h of light per day. All slugs moved to LD 12:12 at hatching showed at least partial development of male parts. However, only four of the 200 eggs that developed under LD 0:24 at 22°C gave rise to females; the rest showed some male development.

Embryogenesis under the other three conditions resulted in a high frequency of females and almost no complete hermaphrodites (Figs. 2–4). Eggs developing under LD 0:24 at 15°C gave rise to a small proportion of androgynous females in four post-hatching groups, but no hermaphrodites occurred, regardless of post-hatching experience (Fig. 2). Eggs developing at the warmer temperature and exposed to a day length of 12 h also tended to have incomplete or no development







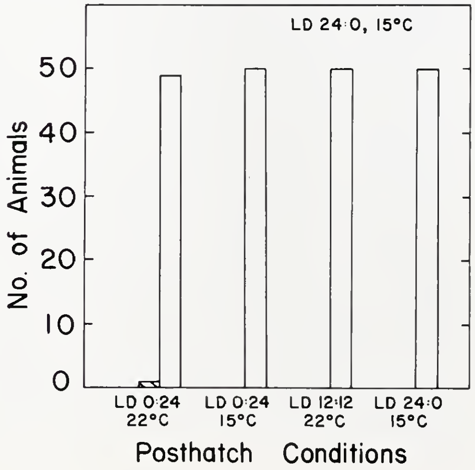


FIGURE 1. (Top left.) Frequencies of three sexual morphs among *Deroceras laeve* exposed to LD 0:24 at 22°C during embryogenesis. At hatching slugs were transferred to the indicated conditions and scored for sexual morphology as adults. Solid bars, hermaphrodites; cross-hatched bars, androgynous females; open bars, females.

FIGURE 2. (Top right.) Frequencies of sexual morphs among *Deroceras laeve* exposed to LD 0:24 at 15°C during embryogenesis and transferred to the indicated conditions at hatching.

FIGURE 3. (Below left.) Frequencies of sexual morphs among *Deroceras laeve* exposed to LD 12:12 at 22°C during embryogenesis and transferred to the indicated conditions at hatching.

FIGURE 4. (Below right.) Frequencies of sexual morphs among *Deroceras laeve* exposed to LD 24:0 at 15°C during embryogenesis and transferred to the indicated conditions at hatching.

of male parts (Fig. 3). Seven hermaphrodites were produced, all of these in the post-hatch group under LD 0:24 at 22°C. Embryonic exposure to light also inhibits development of the penis, as does low temperature. In the last set of eggs (Fig. 4) held under LD 24:0 at 15°C, only one individual showed any sign of male parts, again among slugs reared post-hatching in the dark at 22°C. The remaining 199 females in that set reflect the combined effects of cooler temperature and exposure to light in the inhibition of complete hermaphroditism in laboratory reared *Deroceras laeve*. The general picture suggests that exposure to cool temperatures and light inhibits the development of male organs. Environmental conditions before hatching have the greater effect on adult penis morphology, but post-hatching conditions slightly modify the number of hermaphrodites among eggs developed at warmer temperatures (22°C) (Figs. 1, 3). It is interesting that in these experiments genetically identical individuals exhibited a range of sexual morphologies even when reared in the same container.

Although gonads and spermatheca of ten individuals in each rearing group were inspected under a compound microscope, no evidence of spermatogenesis nor sperm storage was found. Oocytes were common in the gonads of all three morphological types.

Sexual change in a field population

Penis morphology of *Deroceras laeve* varied during the warm season of 1979 in the artificially established field population (Fig. 5). The frequency of females was near 100% until a precipitous decline to 16% in July. In that sample 59% of the adults were hermaphrodites and 25% androgynous females. The number of females increased gradually during the remainder of the season, reaching 50% in November. Androgynous females were found at a low frequency (<25%) in every sample. Samples from this population reflect egg development 4–6 weeks previously. In general, an increasing frequency of hermaphrodites as summer progressed correlates with laboratory experiments on penis morphology: Females developed from eggs laid when temperatures were lower; male parts were produced during warmer weather. The importance of other environmental variables in nature is unknown.

No genetic evidence of outcrossing was found in samples of this population. All individuals were homozygous, with both marker alleles of IDH represented in each sample. While persistence of homozygosity could suggest self-fertilization, by analogy with the laboratory experiments, we think it more likely that it suggests continued asexual reproduction. In support of this, we were unable to detect any evidence of sperm or spermatogenesis in the 1980 samples, even though we have had no problems in visualizing them in *D. reticulatum* by the same technique

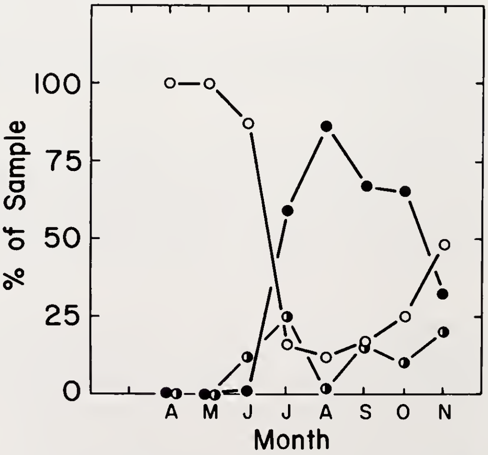


FIGURE 5. Frequencies of three sexual morphs at various times of the year among *Deroceras laeve* in an artificially established field population. Collections were made in 1979. Closed circles, hermaphrodites; half-filled circles, androgynous females; open circles, females.

(unpublished). No obvious relationship was seen between IDH genotype and sexual phenotype. Frequencies of marker alleles were similar among the three phenotypes.

Population size remained relatively stable throughout the season. Numbers of adults (≥0.06 g) discovered in 2 h by manual search of refuges ranged from a low of 48 in April to a high of 69 in June. During each collection period, small slugs and clutches of eggs were found but were left relatively undisturbed.

Electrophoretic analysis of natural populations

Laboratory studies of the mode of inheritance were supplemented with a survey of 10 natural populations of *Deroceras laeve*. Twenty electrophoretic loci of 17 enzymes were scored (Table I). Within eight populations there was no variation in banding pattern; every individual was homozygous for all loci. In one sample (site code CAN) 17 of 52 slugs were heterozygous for EST2 with a two banded phenotype. The remainder were homozygous for the fast allele. This was the only population consisting of more than one clone. From another site one mile distant (TIM) all 30 slugs collected were heterozygous at that locus. These samples were otherwise electrophoretically identical, with homozygosity at all other loci.

Among 10 populations, seven different genotypes were found involving mobility differences at isocitrate dehydrogenase (IDH), malic enzyme (ME), α-glycerophosphate dehydrophase (α-GPDH), and three esterase loci (Table II). Three alleles were found for EST2; the other variable loci had two alleles. Differences between populations generally increased with geographical distance. Genotypes I and II co-occurred at CAN as described above, differing only at EST2. Four populations about one mile distant from each other (Mt. Lebanon, Pa.) were identical at all loci scored (genotype VII). The remaining populations were more than 10 miles apart, and differed from each other at 1–6 loci. Genotype VI was the least similar to any other, differing from the others at 3–6 loci. It also contained alleles for EST2 and IDH not found in any other population. The average number of alleles held in common between populations among the 20 loci studied was 17 (85%), a relatively high degree of similarity compared with other invertebrates (Lewontin, 1974).

TABLE II

Genotypes for the six variable enzyme loci found in 10 populations of Deroceras laeve. *Alleles are named according to electrophoretic mobility, with A the most anodal. Sample sizes in parentheses.*

Genotype:	I	II	II	IV	V	VI	VII
Site code:	CAN (35)	CAN (17) TIM (30)	BRI (21)	SOA (48)	PAS (37)	MAR (62)	MIS (35) HSS (48) BAY (49) MET (50)
Locus:							
EST1	BB	BB	BB	BB	AA	AA	BB
EST2	AA	AB	AA	BB	BB	CC	BB
EST3	AA	AA	AA	AA	AA	BB	BB
IDH	AA	AA	AA	AA	AA	BB	AA
αGPDH	AA	AA	BB	BB	BB	BB	BB
ME	BB	BB	AA	AA	AA	AA	AA

DISCUSSION

The discovery of apomictic parthenogenesis in *Deroceras laeve* represents the first documentation of parthenogenesis in a pulmonate to our knowledge. It may help to explain aspects of the slug's biology not well understood previously. *D. laeve* is believed to have migrated from Asia to North America in a post-glacial incursion (Pilsbry, 1948) and has now spread across the continent to become one of the most common slug species in non-arid regions (Pilsbry, 1948; Chichester and Getz, 1969). This species has a wide tolerance of environmental conditions relative to other species of slugs and begins to reproduce earlier in the spring than the imported *D. reticulatum* (Getz, 1959). Relatively short generation time (6 weeks from egg to egg in the laboratory), parthenogenetic reproduction, and high reproductive potential (up to 350 fertile eggs per slug) are all characteristics typical of colonizing species (Lewontin, 1965). Asexual populations of colonizing species are often characterized by sudden appearances in great numbers and abrupt disappearances, as reported for example among parthenogenetic prosobranchs, *Melanoides* spp. (Jacob, 1958a; Winterbourne, 1970) and *Potamopyrgus jenkinsi* (Jacob, 1958a; Robson, 1923), as well as in the sea anemone, *Haliplanella luciae* (Shick and Lamb, 1977). These snails and anemones are tolerant of wide temperature and salinity ranges, but mortality at the limits of tolerance is precipitous, a function of genetic uniformity. No outcrossing has been reported in these other species.

Since parthenogenesis has a potential genetic advantage over self-fertilization in preserving intact well-adapted genotypes and in avoiding the approach to homozygosity necessary with self-fertilization (Marshall and Weir, 1979), it is somewhat puzzling that most electrophoretic loci in the natural populations that we sampled were homozygous. This sort of population structure would ordinarily be expected from self-fertilization, and, in fact, has been interpreted by McCracken and Selander (1980) as indicating self-fertilization in *Deroceras laeve*, as well as a number of other slug species. Selander and Kaufman (1973) and Selander and Hudson (1976) have also interpreted a similar population structure in a land snail, *Rumina decollata*, as indicative of self-fertilization. Based only on surveys of natural populations without the breeding experiments that we report here, their interpretations seem reasonable. Nonetheless, our finding of a complete lack of segregation in 23 separate families of *D. laeve* reared from eggs laid in the absence of mates is only consistent with apomixis. No other known genetic mechanism could produce such a pattern. It is also important that one natural population that we sampled (TIM) consisted of a single clone heterozygous at the EST2 locus (Table II), a result also incompatible with self-fertilization.

In the case of the data on *D. laeve*, the species for which we have unequivocal evidence of parthenogenesis, there are some possible explanations for the incompatibility of our experimental evidence with the inferences of McCracken and Selander (1980). It is possible that the morphological species *Deroceras laeve* consists of two or more cryptic species and that we are reporting on a parthenogenetic one. Alternatively, there could be variation in reproductive mode among populations. It is also worth noting that one of the esterase loci (EST1) instrumental in our breeding studies is freeze-labile and would go undetected in the frozen samples used by McCracken and Selander (1980). Finally, McCracken and Selander (1980) did not do the breeding necessary to establish unequivocally the mating system of any of the slugs they report on, and, as they point out, they are unable to rule out parthenogenesis as a result.

The production of monogenic clones of *D. laeve* by apomixis, a process that exactly duplicates an entire genotype, remains somewhat mysterious. Such strains should faithfully preserve any accumulated heterozygosity, even if only that produced by mutation. Our results and those of McCracken and Selander (1980) suggest that the heterozygosity expected from this mode of reproduction is not commonly present in natural populations, although we did find one such population (TIM) and a second (CAN) consisting of one monogenic clone and one heterozygous for EST2 (Table II). The genetic consequences of parthenogenesis, self-fertilization, and outcrossing are identical if all individuals in a population are genetically identical and homozygous. On the other hand, the fact that most of the 10 sampled populations in our study consisted of unique uniclonal genotypes that differ from each other may suggest that apomixis serves in this case to preserve locally well-adapted multilocus genotypes, analogous to the situation suggested for some sea anemones by Shick *et al.* (1979).

Our experiments suggest that outcrossing is infrequent in *Deroceras laeve*. None was detected in the biclonal field population constructed during the study. Laboratory stocks made with original pair matings remained heterozygous for the appropriate loci with no appearance of the homozygotes expected from sib matings, even when slugs were reared in mass culture. This suggests that no further outcrossing took place, in spite of repeated opportunity. Pair matings attempted between *D. laeve* of other populations were also unsuccessful. The slugs consistently reproduced parthenogenetically. It is possible that some populations in this study lack the ability to outcross.

The relationship between environmental conditions and development of male parts and male function must determine in part the mode of reproduction of *Deroceras laeve*. These experiments suggest that development of male end organs depends upon temperature and photoperiod. Pilsbry (1948) discusses at some length the morphological variability of natural populations of this species, with some entirely hermaphroditic, others strictly female, and still others mixed. He argues against succession of sexual phases in individuals, since only rare individuals showed partial development of the penis. Pilsbry emphasized that more attention must be paid to the season in which particular morphs are collected. The variation in sexual condition of the artificially established population throughout a season, as reported above, supports the contention that sexual morphology is primarily a result of environmental conditions. Factors leading to outcrossing in this species are unclear. Both hermaphrodites and females can be parthenogenetic. Those slugs which did mate were all hermaphrodites. Females never mated.

In other species of slugs, sexual reproduction is under endrocrinological control triggered by environmental cues, with day length of primary importance (McCrone and Sokolove, 1979; Sokolove and McCrone, 1978). Fertility and fecundity in *Deroceras reticulatum* and *Limax flavus* can be enhanced or inhibited by injection of steroid hormones (Takeda, 1979) or extracts of brain or eyestalks (Takeda, 1977). Detailed histological studies of maturation of reproductive systems in *D. reticulatum* (Runham and Laryea, 1968) and *Arion ater* (Lusis, 1966; Smith, 1966) show that light and humidity exert greater effects on spermatogenesis than oogenesis. Photoperiod is critical in male-phase maturation in *Limax maximus* but has little effect on female-phase maturation (Sokolove and McCrone, 1978). In all of these studies, development of male characteristics is more sensitive to environmental manipulation than is female expression.

Selection for rapid maturation in three species of *Deroceras* capable of reproduction without mating and most pronounced in *D. laeve* (Abeloos, 1945) may have paralled selection for parthenogenesis. Early reproduction contributes to a high reproductive potential. Abeloos (1945) characterized development of *D. laeve* as "precocious" relative to the larger *D. agreste*, with the genus *Deroceras* in turn having fewer discrete growth phases than other limacids or arionids. Studies by Reygrobellet (1963) and Maury and Reygrobellet (1964) may suggest that *D. meridionale* and *D. agreste* are also parthenogenetic, rather than self-fertile, since in those studies discrete "clones" are described for these species as well as for *D. laeve*. Inheritance of strain-specific penis morphology was followed for 10 generations in the laboratory with no opportunity for outcrossing. Genetic analysis of the "clones" was not done, so the mode of inheritance in these species is not known.

Our discovery of both parthenogenesis and outcrossing *Deroceras laeve* extends the diversity of reproductive modes known within the class Gastropoda. Parthenogenesis has been documented in the prosobranchs *Melanoides* spp. and *Potamopyrgus jenkinsi* (Robson, 1923; Jacob, 1958a, 1958b). These species, however, are not known to outcross, consisting almost exclusively of thelytokous females which frequently are polyploid (Jacob, 1958b; Winterbourne, 1970). The majority of gastropods are sexual, but with a variety of reproductive modes. Simultaneous hermaphroditism is common among snails and slugs, *e.g., Deroceras reticulatum* (Pilsbry, 1948; Runham and Hunter, 1970). Some of these species are capable of self-fertilization, carefully documented by Ikeda (1937) for example, in the slug *Philomycus bilineatus*. It will be important to distinguish self-fertilization from parthenogenesis in other species capable of reproduction without outcrossing.

ACKNOWLEDGMENTS

This research was supported by grants T-4 from the Health Research and Services Foundation (Pittsburgh, PA), NSF DEB77-14442, and NIH GM 25809 to R.J.H.

LITERATURE CITED

ABELOOS, M. 1945. Évolution par variation du nombre des phases de croissance chez les Mollusques Limaciens. *C. R. Hebd. Seances Acad. Sci.* **220:** 62–63.

BREWER, G. J. 1970. *An Introduction to Isozyme Techniques*. Academic Press, New York. 186 pp.

CHICHESTER, L. F., AND L. L. GETZ. 1969. The zoogeography and ecology of arionid and limacid slugs introduced into northeastern North America. *Malacologia* **7:** 313–346.

GETZ, L. L. 1959. Notes on the ecology of slugs: *Arion circumscriptus, Deroceras reticulatum* and *D. laeve*. *Am. Midl. Nat.* **61:** 485–498.

HUMASON, G. L. 1972. *Animal Tissue Techniques*. 3rd Ed., W. H. Freeman, San Francisco. 641 pp.

IKEDA, K. 1937. Cytogenetic studies on the self-fertilization of *Philomycus bilineatus* Benson. *J. Sci. Hiroshima Univ.* **B5:** 68–123.

JACOB, J. 1958a. Cytological studies of Melaniidae (Mollusca) with special reference to parthenogenesis and polyploidy. I. Oogenesis of the parthenogenetic species of *Melanoides* (Prosobranchia-Gasteropoda). *Trans. R. Soc. Edinb.* **63:** 341–352.

JACOB, J. 1958b. Cytological studies of Melaniidae (Mollusca) with special reference to parthenogenesis and polyploidy. II. A study of meiosis in the rare males of the polyploid race of *Melanoides tuberculatus* and *M. lineatus*. *Trans. R. Soc. Edinb.* **63:** 433–445.

LEWONTIN, R. C. 1965. Selection for colonizing ability. Pp. 77–94 in H. G. Baker and G. L. Stebbins, Eds., *The Genetics of Colonizing Species*. Academic Press, New York.

LEWONTIN, R. C. 1974. *The Genetic Basis of Evolutionary Change*. Columbia Univ. Press, New York. 346 pp.

LUSIS, O. 1966. Changes induced in the reproductive system of *Arion ater rufus* L. by varying environmental conditions. *Proc. Malacol. Soc. Lond.* **37:** 19–26.

MARSHALL, D. R., AND B. S. WEIR. 1979. Maintenance of genetic variation in apomictic plant populations. I. Single locus models. *Heredity* **42:** 159–172.

MAURY, M. F., AND D. REYGROBELLET. 1964. Sur les distinctions specifiques chez les Mollusques Limacides du genre *Deroceras*. *C. R. Hebd. Seances Acad. Sci.* **257:** 275–277.

MAYNARD SMITH, J. 1978. *The Evolution of Sex.* Cambridge Univ. Press, Cambridge. 222 pp.

MCCRACKEN, G. F., AND R. K. SELANDER. 1980. Self-fertilization and monogenic strains in natural populations of terrestrial slugs. *Proc. Natl. Acad. Sci. USA.* **77:** 684–688.

MCCRONE, E. J., AND P. G. SOKOLOVE. 1979. Brain-gonad axis and photoperiodically-stimulated sexual maturation in the slug. *Limax maximus. J. Comp. Physiol.* **133:** 117–123.

NICHOLS, E. A., AND F. H. RUDDLE. 1973. A review of enzyme polymorphism, linkage and electrophoresis conditions for mouse and somatic cell hybrids in starch gels. *J. Histochem. Cytochem:* **21,** 1066–1081.

PILSBRY, H. A. 1948. *Land Mollusca of North America.* Vol. 2, part 2. The Academy of Natural Sciences of Philadelphia, Philadelphia, Pa.

REYGROBELLET, D. 1963. Une novelle espece de Limacide, *Deroceras meriodionale* n. sp. *Bull. Soc. Zool. Fr.* **88:** 399–402.

ROBSON, G. C. 1923. Parthenogenesis in the mollusc *Paludestrina jenkinsi. J. Exp. Biol.* **1:** 65–78.

RUNHAM, N. W., AND P. J. HUNTER. 1970. *Terrestrial Slugs.* Hutchinson Univ. Library, London. 184 pp.

RUNHAM, N. W., AND A. A. LARYEA. 1968. Studies on the maturation of the reproductive system of *Agriolimax reticulatus* (Pulmonata: Limacidae). *Malacologia* **7:** 93–108.

SELANDER, R. K., AND R. O. HUDSON. 1976. Animal population structure under close inbreeding in the land snail *Rumina* in Southern France. *Am. Nat.* **110:** 695–718.

SELANDER, R. K., AND D. W. KAUFMAN. 1973. Self-fertilization and genetic population structure in a colonizing land snail. *Proc. Natl. Acad. Sci. USA.* **70:** 1186–1190.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON, AND J. B. GENTRY. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus.* I. Variation in the old field mouse (*Peromyscus polionotus*). *Studies in Genetics IV,* Univ. Texas Publ. 7103, pp. 49–90.

SHAW, C. R., AND R. PRASAD. 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. *Biochem. Genet.* **4:** 297–320.

SHICK, J. M., R. J. HOFFMANN, AND A. N. LAMB. 1979. Asexual reproduction, population structure, and genotype-environment interactions in sea anemones. *Am. Zool.* **19:** 699–713.

SHICK, J. M., AND A. N. LAMB. 1977. Asexual reproduction and genetic population structure in the colonizing sea anemone *Haliplanella luciae. Biol. Bull.* **153:** 604–617.

SMITH, B. J. 1966. Maturation of the reproductive tract of *Arion ater* (Pulmonata: Arionidae). *Malacologia* **4:** 325–349.

SOKAL, R. R. , AND F. J. ROHLF. 1969. *Biometry.* W. H. Freeman, San Francisco. 776 pp.

SOKOLOVE, P. G., AND E. J. MCCRONE. 1978. Reproductive maturation of the slug, *Limax maximus,* and the effects of artificial photoperiod. *J. Comp. Physiol.* **125:** 317–325.

TAKEDA, N. 1977. Stimulation of egg laying by nerve extracts in slugs. *Nature* **267:** 513–514.

TAKEDA, N. 1979. Induction of egg laying by steriod hormones in slugs. *Comp. Biochem. Physiol.* **62A:** 273–278.

WILLIAMS, G. C. 1975. *Sex and Evolution.* Princeton Univ. Press, Princeton, New Jersey. 200 pp.

WINTERBOURNE, M. 1970. The New Zealand species of *Potamopyrgus* (Gasteropoda: Hydrobiidae). *Malacologia* **10:** 283–321.

Wds Hole

Reference: *Biol. Bull.*, **160:** 136–145. (February, 1981)

L1

SELECTIVE PREDATION BY *FAVELLA EHRENBERGII* (TINTINNIA) ON AND AMONG DINOFLAGELLATES[1]

DIANE STOECKER, R. R. L. GUILLARD, AND RHONDA M. KAVEE[2]

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

ABSTRACT

In culture, the tintinnid *Favella ehrenbergii* requires dinoflagellates as food. Of the dinoflagellates tested, strain Gymno, *Gonyaulax tamarensis, G. polyedra*, and *Heterocapsa* sp. are good foods and *Prorocentrum mariaelebouriae* is a poor food. *Amphidinium carterae*, which produces choline-like substances, is not eaten.

Favella recognizes dinoflagellates with very different sizes and morphologies as prey. In mixtures of dinoflagellates and non-dinoflagellates, *Favella* selectively preys on dinoflagellates. Cryptophytes, haptophytes, chrysophytes, diatoms, prasinophytes, and chlorophytes of suitable size are consumed in small amounts, if at all.

INTRODUCTION

Tintinnids (ciliated protozoa, suborder Tintinnia) are a major component of microzooplankton (Beers and Stewart, 1967, 1969, 1971; Johansen, 1976) and are important predators on nanophytoplankton (Blackbourn, 1974; Johansen, 1976; Heinbokel and Beers, 1979). Spittler (1973) and Heinbokel (1978) found that tintinnids ingest only particles with diameters less than 42–45% of the tintinnids' oral lorica diameters. Ciliates, including tintinnids, are assumed to select food primarily by size (Rassoulzadegan, 1978; Heinbokel and Beers, 1979; Fenchel, 1980). However, differential predation by some tintinnids on similar-sized particles has been observed (Spittler, 1973; Blackbourn, 1974; Johansen, 1976; Heinbokel, 1978).

Tintinnids in the genus *Favella* are often associated with dinoflagellate blooms. In the Bay of Fundy, *Favella* sp. prey on the dinoflagellate *Gonyaulax tamarensis* (= *Gonyaulax excavata* of some authors) and coincide in abundance with dinoflagellates (Needler, 1949; Prakash, 1963; White, 1979). Blackbourn (1974) observed that *F. serrata* was usually associated with high dinoflagellate numbers in British Columbia. We isolated a strain of *F. ehrenbergii* (Clap. and Lach.) Jorg. from a bloom of the dinoflagellate *Prorocentrum* sp. (similar to *P. micans*) in Boston Harbor, Massachusetts, and have observed *F. ehrenbergii* during blooms of the dinoflagellates *Heterocapsa* sp. and *G. tamarensis* in salt ponds on Cape Cod, Massachusetts. We found that *F. ehrenbergii* could only be cultured if dinoflagellates were in the algal food mixture. Gold (1969) similarly observed that *F. campanula* could only be cultured if dinoflagellates were in the diet. These observations suggested to us that *Favella* may be a specialized predator on dino-

---

Received 23 July 1980, accepted 20 November 1980.

Abbreviations: SWT: 1.0 μM filtered seawater with 0.01–0.05 ml/l of f/2 iron EDTA trace metal solution (Guillard, 1975).

[1] Contribution no. 4746 from the Woods Hole Oceanographic Institution. This research was supported by the Department of Commerce, NOAA Office of Sea Grant under Grant No. NA79AA-D-00102. D. Stoecker held a Woods Hole Oceanographic Institution Post-Doctoral Scholarship and a Leopold Schepp Foundation Post-Doctoral Fellowship.

[2] Present address: 60 Vine Road, Larchmont, NY 10538.

flagellates or may require certain dinoflagellates in its diet. To test these hypotheses, we fed *F. ehrenbergii* on monocultures of various dinoflagellates, on mixtures of two dinoflagellates, and on mixtures consisting of a dinoflagellate and a member of another algal group.

MATERIALS AND METHODS

Culture of phytoplankton

The algal species listed in Table I were used in feeding experiments. All autotrophic species were grown in enriched seawater medium f/2 (Guillard, 1975) except that silicic acid was omitted for non-diatoms and ammonium added for clones ϕ and θ (medium "h/2," Guillard, 1975). The heterotrophic *Crypthecodinium cohnii* was grown in medium h/2 with organic enrichment "II_c" (Guillard, 1960). All cultures were grown on a 14:10 h light-dark cycle under *ca.* $2.5 \cdot 10^{-2}$ langley$\cdot$min^{-1} of Cool-White (Sylvania Co.) fluorescent light at 20°C, except that *Gonyaulax tamarensis* and *Cachonina niei* were kept at 15°C. We fed only log phase algal cultures. We measured phytoplankton cell size with an occular micrometer.

Isolation and culture of Favella

All glass or plastic utensils were autoclaved filled with distilled water to remove trace contaminants. The culture medium (SWT) was 1.0 μM filtered Vineyard Sound seawater to which 0.01–0.05 ml/l of the f/2 iron-EDTA trace metal solution (Guillard, 1975) was added. This was autoclaved in teflon, cooled, and later poured into appropriate culture vessels. All cultures were kept on a 14:10 h light cycle and transferred weekly.

Strain BH-FAV of *F. ehrenbergii* was isolated from a surface water sample collected in Boston Harbor, Massachusetts on 3 October 1979. The isolated tintinnids initially were grown in wells of a borosilicate glass spot plate containing 0.5 ml of SWT. One to three individuals were placed in each well. Phytoplankton cells at concentrations ranging from about 10/ml to about 10^5/ml were added. Twelve non-dinoflagellates, *Synechococcus* sp., *Chroomonas salina, Isochrysis galbana, Pavlova lutheri, Thalassiosira pseudonana, Asterionella glacialis, Olisthodiscus luteus, Platymonas* sp., *Micromonas* sp. (*pusilla*), *Nannochloris* sp., *Stichococcus* sp., and *Dunaliella tertiolecta*, and one dinoflagellate, Strain Gymno, were tried as food. Three replicate tests were made. The spot plates were incubated in closed, clear plastic containers at 15°C or 20°C. After 2 days, the tintinnids had survived and reproduced only in the wells containing Gymno.

The tintinnids that grew in the wells were then transferred to 250 ml polycarbonate culture vessels containing 50–100 ml of SWT. Routinely, *F. ehrenbergii* were fed a mixture containing 1×10^4 cells/ml of Gymno and small amounts of *Chroomonas salina* and *Isochrysis galbana.*

Growth of F. ehrenbergii *on algal monocultures*

Experiments designed to determine the species and concentrations of dinoflagellates that would best support *F. ehrenbergii* growth in culture were conducted in culture flasks containing 50 ml of sterilized SWT with initial concentrations of algae from 10/ml to 5×10^4/ml. We added 25 tintinnids to each of three replicate flasks for each algal species and concentration and counted the number of tintinnids in each flask after 4 days.

TABLE I

Algal species used in feeding experiments

Species	Strain	Approximate Dimensions (μm)
Cryptophytes		
Chroomonas salina (Wislouch.) Butcher	3C	6 × 12
Unidentified sp.	ϕ	5 × 7
Unidentified sp.	θ	5 × 7
Dinoflagellates		
Small *Gymnodinium*-like dinoflagellate	Gymno	7 × 14
Prorocentrum mariaelebouriae (Parke and Ballantine) Loeblich III	Exuv	12 × 18
Crypthecodinium cohnii (Seligo) Chatton in Grasse	*C. cohnii*	16 × 17
Thoracosphaera heimii (Lohm.) Kampt.	A603	11–15*
Amphidinium höfleri Schiller and Diskus**	*A. hoef.*	7 × 15
Amphidinium carterae Hulburt	Amphi	10 × 16
Zooxanthella microadriaticum Freudenthal	*T. gigas*	7 × 15
Cachonina niei Loeblich III	*C. niei*	22 × 25
Scrippsiella trochoidea (Stein) Loeblich III	Peri	23 × 30
Gonyaulax polyedra F. Stein	GP60e	34 ± 6***
Heterocapsa sp.	HT984	16 × 22
Gonyaulax tamarensis Lebour	GT429	32 ± 7***
Haptophytes		
Hymenomonas carterae (Braarud & Fagerl.) Braarud	Cocco II	10 × 10
Unidentified sp.	H. H.	4 × 10
Diatoms		
Thalassiosira weissflogii (Grunow) Frywell & Hasle (ex. *T. fluvialis*)	Actin	10 × 15
Thalassiosira pseudonana (Hurst.) Hasle & Heimdal	13-1	7 × 7
Cyclotella cryptica Reiman, Lewin, & Guillard	WT-1-8	11 × 12
Chrysophytes		
Olisthodiscus luteus Carter	Olisth	7 × 15
Prasinophytes		
Platymonas sp.	Platy I	7 × 10
Pyraminonas sp.	Pyr 1	7 × 10
Pyraminonas sp.	Pyr 2	6 × 7
Chlorophytes		
Chlamydomonas sp.	D	12–18*
Dunaliella tertiolecta Butcher	Dun	5 × 11

* Range of diameters.

** See Taylor (1971) for a discussion of the uncertain taxonomic position of this species.

*** Greatest diameter ± SD.

Selective feeding experiments

Feeding choice experiments were designed to determine if *F. ehrenbergii* would prey preferentially on one algal species in mixtures of two. In experiments with *Gonyaulax tamarensis* and *G. polyedra* as prey, about 1×10^2 cells/ml of each

TABLE II

Growth of Favella ehrenbergii *on dinoflagellate monocultures at optimal initial algal cell densities. Mean* Favella *count* ± *SD with calculated* Favella *density (cells/ml) in parentheses.*

Species	Optimal Initial Algal Density (cells/ml)	*Favella* Abundance[a]
Amphidinium carterae	—	0
Strain Gymno	1×10^4	150 ± 36 (3.0)
Gonyaulax tamarensis	1×10^2	356 ± 165 (7.1)
Gonyaulax polyedra	1×10^2	287 ± 40 (5.7)
Scrippsiella trochoidea	5×10^2	421 ± 20 (8.4)
Heterocapsa sp.	1×10^3	507 ± 33 (10.1)
Prorocentrum mariaelebouriae	1×10^3	55 ± 7 (1.1)

[a] Initial *Favella* density was 25/50 ml (0.5/ml). *Favella* counted after 4 days incubation at 20° (except 15° with *G. tamarensis*).

species was used. With *Scrippsiella trochoidea* and *Cachonina niei*, about 5×10^2 cells/ml of each species was used. In all other feeding choice experiments (i.e. with algae about the size of strain Gymno.), 1×10^4 cells/ml of each alga was used. All experiments were run in triplicate in 5 ml of SWT in 25 × 50 mm Pyrex glass

TABLE III

Algal cell counts in mixtures of dinoflagellates subject to grazing by Favella. *Upper number is the mean cell count* ± *SD. Lower number (in parentheses) is the calculated number of cells per ml.*

No. of *Favella*/ml	Strain Gymno	*Prorocentrum* sp.	Strain Gymno	*C. cohnii*	Strain Gymno	*T. heimii*
0	73 ± 8 (1.5×10^3)	73 ± 10 (1.5×10^3)	134 ± 32 (1.3×10^4)	108 ± 16 (1.1×10^4)	335 ± 18 (0.7×10^4)	578 ± 20 (1.2×10^4)
5	54 ± 6 (1.1×10^3)	56 ± 6 (1.1×10^3)	104 ± 6 (1.0×10^4)	82 ± 1 (0.8×10^4)	262 ± 23 (0.3×10^4)	381 ± 20 (0.8×10^4)
10	43 ± 4 (0.9×10^3)	43 ± 5 (0.9×10^3)	66 ± 10 (0.6×10^4)	66 ± 15 (0.7×10^4)	211 ± 6 (0.1×10^4)	262 ± 10 (0.5×10^4)
	Strain Gymno	*A. höfleri*	Strain Gymno	*A. carterae*	Strain Gymno	*Z. microadriaticum*
0	198 ± 13 (1.9×10^4)	76 ± 2 (0.5×10^4)	114 ± 2 (2.3×10^3)	130 ± 6 (2.6×10^3)	102 ± 11 (1.0×10^4)	111 ± 14 (1.1×10^4)
5	160 ± 7 (1.6×10^4)	39 ± 4 (0.3×10^4)	78 ± 4 (1.6×10^3)	130 ± 8 (2.6×10^3)	65 ± 5 (0.6×10^4)	66 ± 2 (0.7×10^4)
10	139 ± 4 (1.4×10^4)	28 ± 2 (0.2×10^4)	69 ± 3 (1.4×10^3)	130 ± 12 (2.6×10^3)	49 ± 2 (0.5×10^4)	51 ± 11 (0.5×10^4)
	C. niei	*S. troichoidea*	*G. polyedra*	*Heterocapsa* sp.	*G. tamarensis*	*Heterocapsa* sp.
0	132 ± 18 (1.3×10^3)	231 ± 12 (0.5×10^3)	131 ± 4 (1.3×10^2)	119 ± 7 (1.2×10^2)	129 ± 4 (1.3×10^2)	209 ± 2 (2.1×10^2)
5	87 ± 4 (0.9×10^3)	147 ± 18 (0.3×10^3)	80 ± 14 (0.8×10^2)	89 ± 12 (0.9×10^2)	64 ± 5 (0.6×10^2)	155 ± 3 (1.6×10^2)
10	64 ± 6 (0.6×10^3)	111 ± 10 (0.2×10^3)	57 ± 3 (0.6×10^2)	78 ± 2 (0.8×10^2)	53 ± 4 (0.5×10^2)	129 ± 10 (1.3×10^2)

TABLE IV

Algal cell counts in mixtures of dinoflagellates and cryptophytes subject to grazing by Favella. *Upper number is the mean cell count ± SD. Lower number (in parentheses) is the calculated number of cells per ml.*

No. of *Favella*/ml	Strain Gymno	*C. salina*	Strain Gymno	Strain ϕ	Strain Gymno	Strain θ
0	398 ± 13 (8.0×10^3)	272 ± 9 (5.4×10^3)	89 ± 2 (1.8×10^3)	147 ± 22 (3.0×10^3)	202 ± 5 (2.0×10^4)	309 ± 8 (3.1×10^4)
5	195 ± 6 (3.9×10^3)	253 ± 12 (5.1×10^3)	57 ± 4 (1.1×10^3)	136 ± 16 (2.8×10^3)	136 ± 11 (1.3×10^4)	318 ± 9 (3.2×10^4)
10	105 ± 6 (2.1×10^3)	256 ± 9 (5.1×10^3)	40 ± 5 (0.8×10^3)	150 ± 17 (3.0×10^3)	65 ± 3 (0.6×10^4)	301 ± 6 (3.0×10^4)

culture tubes incubated in light for 8 h at 20°C; except that *C. niei* and *G. tamarensis* were incubated at 15°C. Control tubes contained no tintinnids, experimental tubes contained 5 or 10 tintinnids/ml. After incubation, the contents of the tubes were fixed with Lugol's solution and algal densities determined using appropriate counting chambers for the cell sizes and densities (Guillard, 1973).

RESULTS

Algal monoculture experiments

Favella ehrenbergii survived and reproduced when fed monocultures of the dinoflagellates Strain Gymno, *Gonyaulax tamarensis, Scrippsiella trochoidea, Gonyaulax polyedra, Heterocapsa* sp., and *Prorocentrum mariaelebouriae*; but did not survive when fed the dinoflagellate *Amphidinium carterae* (Table II). *P. mariaelebouriae* was a poor food; *Favella* population densities were considerably lower when tintinnids were fed this dinoflagellate than when fed Strain Gymno, *G. tamarensis, S. trochaidea, G. polyedra*, or *Heterocapsa* sp. (Table II).

Selective feeding experiments

In the cultures containing two dinoflagellates, *Favella* preyed on both species, except when one of them was *A. carterae*, which *Favella* did not consume (Table III). In the cultures containing a dinoflagellate and a non-dinoflagellate, the di-

TABLE V

Algal cell counts in mixtures of dinoflagellates and haptophytes subject to grazing by Favella. *Upper number is the mean cell count ± SD. Lower number (in parentheses) is the calculated number of cells per ml.*

No. of *Favella*/ml	Strain Gymno	*H. carterae*	Strain Gymno	Strain H.H.
0	120 ± 4 (1.2×10^4)	130 ± 6 (1.3×10^4)	80 ± 7 (0.8×10^4)	178 ± 6 (1.8×10^4)
5	96 ± 2 (1.0×10^4)	126 ± 5 (1.3×10^4)	53 ± 4 (0.5×10^4)	183 ± 10 (1.8×10^4)
10	80 ± 6 (0.8×10^4)	127 ± 6 (1.3×10^4)	42 ± 3 (0.4×10^3)	186 ± 14 (1.9×10^4)

Table VI

Algal cell counts in mixtures of dinoflagellates and diatoms subject to grazing by Favella. *Upper number is the mean cell count* ± *SD. Lower number (in parentheses) is the calculated number of cells per ml.*

No. of *Favella*/ml	Strain Gymno	*C. cryptica*	Strain Gymno	*T. weissflogii*	Strain Gymno	*T. pseudonana*
0	103 ± 3 (10.3×10^3)	126 ± 18 (2.5×10^3)	90 ± 1 (1.8×10^4)	207 ± 30 (4.1×10^4)	81 ± 2 (5.3×10^3)	115 ± 6 (7.5×10^3)
5	80 ± 6 (8.0×10^3)	129 ± 14 (2.6×10^3)	80 ± 1 (1.6×10^4)	214 ± 13 (4.2×10^4)	54 ± 3 (3.5×10^3)	112 ± 5 (7.3×10^3)
10	38 ± 3 (3.8×10^3)	127 ± 16 (2.6×10^3)	50 ± 5 (1.0×10^4)	202 ± 5 (4.0×10^4)	31 ± 2 (2.0×10^3)	115 ± 6 (7.5×10^3)

noflagellate (Strain Gymno) was always consumed (Tables IV–IX). There was little if any predation on cryptophytes (Table IV), haptophytes (Table V), diatoms (Table VI), a chrysophyte (Table VII), prasinophytes (Table VIII), or chlorophytes (Table IX).

Dependence of predation on algal species in the selective feeding experiments was tested using R × C (rows times columns) tests of independence with the *G* statistic (Sokal and Rohlf, 1969). Predation was independent of algal species in four of the dinoflagellate mixed cultures: Strain Gymno with either *P. mariaelebouriae, Crypthecodinium cohnii*, or *Zooanthella microadriaticum*, and *Cachonina niei* with *S. trochoidea* (Table X). In another four dinoflagellate mixed cultures, the larger dinoflagellate of the pair was preferred: *Thoracosphaera heimii* and *Amphidinium hofleri* were preferred over Strain Gymno; *G. polyedra* and *G. tamarensis* were preferred over *Heterocapsa* sp. (Table X). Tests of independence confirmed the preference of *F. ehrenbergii* for dinoflagellates over non-dinoflagellates (Table X).

Discussion

Favella ehrenbergii is a specialized predator on dinoflagellates and consumes few, if any, of the non-dinoflagellates tested. This tintinnid does not select dinoflagellates over other phytoplankters on the basis of size alone; dinoflagellates ranging in size from Strain Gymno to *G. tamarensis* are consumed, whereas similar

Table VII

Algal cell counts in a mixture of dinoflagellates and chrysophytes subject to grazing by Favella. *Upper number is the mean cell count* ± *SD. Lower number (in parentheses) is the calculated number of cells per ml.*

No. of *Favella*/ml	Strain Gymno	*O. luteus*
0	210 ± 6 (2.1×10^4)	44 ± 8 (4.4×10^3)
5	132 ± 6 (1.3×10^4)	42 ± 6 (4.2×10^3)
10	106 ± 4 (1.0×10^4)	41 ± 1 (4.1×10^3)

Table VIII

Algal cell counts in mixtures of dinoflagellates and prasinophytes subject to grazing by Favella. *Upper number is the mean cell count ± SD. Lower number (in parentheses) is the calculated number of cells per ml.*

No. of *Favella*/ml	Strain Gymno	*Platymonas* sp.	Strain Gymno	*Pyraminonas* sp. (Strain Pyr 1)	Strain Gymno	*Pyraminonas* sp. (Strain Pyr 2)
0	68 ± 6 (6.7×10^3)	129 ± 16 (1.3×10^4)	113 ± 4 (7.4×10^3)	64 ± 3 (4.2×10^3)	87 ± 7 (8.5×10^3)	179 ± 8 (1.8×10^4)
5	47 ± 7 (4.6×10^3)	132 ± 10 (1.3×10^4)	83 ± 3 (5.4×10^3)	64 ± 5 (4.2×10^3)	63 ± 4 (6.2×10^3)	193 ± 20 (1.9×10^4)
10	34 ± 3 (3.3×10^3)	130 ± 3 (1.3×10^4)	70 ± 3 (4.6×10^3)	65 ± 4 (4.2×10^3)	40 ± 3 (4.0×10^3)	174 ± 4 (1.7×10^4)

sized non-dinoflagellates are not. The wide size range of dinoflagellates eaten is consistent with Rassoulzadegan's (1978) observations of the particle size selectivity of *F. ehrenbergii* in the Mediterranean Sea. However, in the choice experiments, the larger dinoflagellate of the pair was usually preferred (Table X). This preference may be because *Favella* encounters the species with the larger cross-sectional diameter more often.

Favella preys on both thecate and non-thecate dinoflagellates and is able to recognize *Thoracosphaera heimii* as a dinoflagellate. *T. heimii* was named as a coccolithophore because of its calcareous test (Lohmann, 1902; Kamptner, 1927). Only recently has tabulation of the internal structure of its shell suggested dinoflagellate affinities (Futterer, 1976; Jafar, 1977). The nuclear structure, morphology of the flagellate stage, and pigment composition, studied in cultured cells, confirm that *T. heimii* indeed is a dinoflagellate (L. Brand *et al.*, Woods Hole Oceanographic Institution, unpublished). *Favella* does not eat *Hymenomonae carterae*, which also has a calcerous test and is spherical like *T. heimii*. In culture, *F. ehrenbergii* will feed on dinoflagellates it would not be likely to encounter in plankton, such as *Crypthecodinium cohnii*, a non-photosynthetic dinoflagellate, and a strain of *Zooxanthella microadriaticum* symbiont in giant clams (Taylor, 1969). *F. ehrenbergii* did not reject *P. mariaelebouriae* in choice experiments, although this strain is a poor food.

F. ehrenbergii rejected *Amphidinium carterae* (Strain Amphi). This dinoflagellate is known to produce choline-like substances (Wangersky and Guillard, 1960;

Table IX

Algal cell counts in mixtures of dinoflagellates and chlorophytes subject to grazing by Favella. *Upper number is the mean cell count ± SD. Lower number (in parentheses) is the calculated number of cells per ml.*

No. of *Favella* sp.	Strain Gymno	*Clamydomonas* sp.	Strain Gymno	*Dunaliella* sp.
0	62 ± 2 (6.3×10^3)	96 ± 2 (9.4×10^3)	79 ± 2 (1.6×10^3)	175 ± 4 (3.5×10^3)
5	49 ± 2 (4.8×10^3)	111 ± 1 (10.9×10^3)	59 ± 5 (1.2×10^3)	167 ± 9 (3.3×10^3)
10	30 ± 3 (2.9×10^3)	90 ± 3 (8.9×10^3)	38 ± 5 (0.8×10^3)	169 ± 10 (3.4×10^3)

Table X

Summary of Favella *grazing on mixtures of algal species. Data analyzed using $R \times C$ tests of independence (association) using the* G *statistic (Sokal and Rohlf, 1969). (n.s. = not significant). Cell counts in the replicate culture tubes were pooled for this analysis.*

Algal Mixture	*G*	Level of Significance	*Favella's* Preference
Dinoflagellates			
Strain Gymno & *P. mariaelebouriae*	0.044	n.s.	none
Strain Gymno & *C. cohnii*	2.458	n.s.	none
Strain Gymno & *T. heimi*	25.796	$p < 0.005$	*T. heimii*
Strain Gymno & *A. höfleri*	16.772	$p < 0.005$	*A. höefleri*
Strain Gymno & *A. carterae*	23.704	$p < 0.005$	Gymno
Strain Gymno & *S. microadriaticum*	0.334	n.s.	none
C. nie & *S. troichoidea*	0.162	n.s.	none
G. polyedra & *Heterocapsa* sp.	11.618	$p < 0.005$	*G. polyedra*
G. tamarensis & *Heterocapsa* sp.	20.720	$p < 0.005$	*G. tamarensis*
Dinoflagellates & Cryptophytes			
Strain Gymno & *C. salina*	274.82	$p < 0.005$	Gymno
Strain Gymno & ϕ	42.996	$p < 0.005$	Gymno
Strain Gymno & θ	152.848	$p < 0.005$	Gymno
Dinoflagellates & Haptophytes			
Strain Gymno & *H. carterae*	137.712	$p < 0.005$	Gymno
Strain Gymno & Strain H. H.	33.306	$p < 0.005$	Gymno
Dinoflagellates & Diatoms			
Strain Gymno & *C. cryptica*	64.866	$p < 0.005$	Gymno
Strain Gymno & *T. weissflogii*	15.794	$p < 0.005$	Gymno
Strain Gymno & *T. pseudonana*	29.460	$p < 0.005$	Gymno
Dinoflagellates & Chrysophytes			
Strain Gymno & *O. luteus*	13.298	$p < 0.005$	Gymno
Dinoflagellates & Prasinophytes			
Strain Gymno & *Platymonas* sp.	26.758	$p < 0.005$	Gymno
Strain Gymno & *Pyraminonas* sp. (Pyr 1)	14.554	$p < 0.005$	Gymno
Strain Gymno & *Pyraminonas* sp. (Pyr 2)	36.410	$p < 0.005$	Gymno
Dinoflagellates & Chlorophytes			
Strain Gymno & *Clamydomonas* sp.	23.224	$p < 0.005$	Gymno
Strain Gymno & *Dunaliella* sp.	29.348	$p < 0.005$	Gymno

Thurburg and Sasner, 1973; Taylor *et al.*, 1974) which Wangersky and Guillard (1960) suggested are defenses against predation. However, Blackbourn (1974) found that *Favella serrata* consumes *Amphidinium carterae* (presumably the same strain). The difference between our results and those of Blackbourn presumably result from differences between *F. ehrenbergii* and *F. serrata* or from differences in culture methods.

Other members of the genus *Favella* are also associated with dinoflagellates, but some *Favella* species are not as specialized as *F. ehrenbergii*. Though *F. serrata* is usually associated with high dinoflagellate densities, it will eat cryptophytes, haptophytes, and chlorophytes as well as dinoflagellates (Blackbourn, 1974). *F. campanula* consumes *Rhodomonas* (a cryptophyte) and *Platymonas* (a prasinophyte) although dinoflagellates are a necessary component of its diet (Gold, 1969).

Tintinnids may be more sensitive to changes in the species composition of phytoplankton than are many larger zooplankters. Many tintinnid species vary greatly in abundance over short periods (Hedin, 1975; Gold and Morales, 1975; Johansen, 1976). Their fast generation times (Gold, 1970, 1971) and ability to encyst (Reid and John, 1978; Paranjape, 1980) may allow many tintinnid species to specialize on particular taxonomic groups of algae; thus *Tintinnopsis subacuta* is associated with euglenoids and *Tintinnidium mucicola* with cryptophytes (Blackbourn, 1974). We have observed cyst formation in *F. ehrenbergii* cultures and consider it likely, though as yet unproved, that this is a method of synchronizing *F. ehrenbergii*'s life history with that of its dinoflagellate prey.

In agreement with Blackbourn (1974) we think that predictions of the impact of tintinnid predation on natural assemblages of phytoplankton cannot be based solely on a knowledge of the size distribution of phytoplankton. Selective feeding by tintinnids may greatly affect the population dynamics of particular phytoplankton species.

ACKNOWLEDGMENTS

We thank D. M. Anderson, L. Brand, I. J. Pintner, L. Provasoli, and B. L. Woodward for their advice and assistance. We thank D. M. Anderson for cultures of *G. tamarensis* and *Heterocapsa*, C. Beam for a culture of *C. cohnii*, L. Brand for a culture of *T. heimii*, and I. J. Pintner and L. Provasoli for cultures of *A. hoefleri, Z. microadriaticum*, and *C. niei*. We appreciate J. J. Lee's encouragement, advice, and comments on the manuscript.

LITERATURE CITED

BEERS, J. R., AND G. L. STEWART. 1967. Micro-zooplankton in the euphotic zone at five locations across the California current. *J. Fish. Res. Bd. Can.* **24:** 2053–2068.

BEERS, J. R., AND G. L. STEWART. 1969. Micro-zooplankton and its abundance relative to the larger zooplankton and other seston components. *Mar. Biol.* **4:** 182–189.

BEERS, J. R., AND G. L. STEWART. 1971. Micro-zooplankters in the plankton communities of the upper waters of the eastern tropical Pacific. *Deep Sea Res.* **18:** 861–883.

BLACKBOURN, D. J. 1974. *The feeding biology of tintinnid protozoa and some other inshore microzooplankton.* Ph.D. Thesis, The Univ. of British Columbia. 224 pp.

FENCHEL, T. 1980. Suspension feeding in ciliated protozoa: Functional response and particle size selection. *Microb. Ecol.* **6:** 1–11.

FUTTERER, D. 1976. Calcerous dinoflagellates ("Calciodinelloideae") and the taxonomic position of the Thoracosphaeroideae. *N. Jb. Geol. Palaont. Abh.* **151:** 119–141.

GOLD, K. 1969. Tintinnida: Feeding experiments and lorica development. *J. Protozool.* **16:** 507–509.

GOLD, K. 1970. Cultivation of marine ciliates (Tintinnida) and heterotrophic flagellates. *Helgol. Wiss. Meeresunters.* **20:** 264–271.

GOLD, K. 1971. Growth characteristics of the mass-reared tintinnid *Tintinnopsis beroidea. Mar. Biol.* **8:** 105–108.

GOLD, K., AND E. A. MORALES. 1975. Seasonal changes in lorica sizes and the species of Tintinnida in the New York Bight. *J. Protozool.* **22:** 520–528.

GUILLARD, R. R. L. 1960. The mutant of *Chlamydononas moewusii* lacking contractile vacuoles. *J. Protozool.* **7:** 262–268.

GUILLARD, R. R. L. 1973. Chapter 19: Division rates. Pp. 289–311 in J. R. Stein, Ed., *Handbook of phycological methods—culture methods and growth measurements.* Cambridge Univ. Press, Cambridge, England.

GUILLARD, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates. Pp. 29–60 in

W. L. Smith and M. H. Chanley, Eds., *Culture of marine invertebrate animals.* Plenum Pub., New York.

Hedin, H. 1975. On the ecology of tintinnids on the Swedish West Coast. *Zoon.* **3:** 125–140.

Heinbokel, J. F. 1978. Studies on the functional role of tintinnids in the Southern California Bight. I. Grazing and growth rates in laboratory cultures. *Mar. Biol.* **47:** 177–189.

Heinbokel, J. F., and J. R. Beers. 1979. Studies on the functional role of tintinnids in the Southern California Bight. III. Grazing impact of natural assemblages. *Mar. Biol.* **52:** 23–32.

Jafar, S. A. 1977. The nature of problematical microfossils of the English Gault. *N. Jb. Geol. Palaontol. Abh.* **153:** 180–192.

Johansen, P. L. 1976. A study of tintinnids and other protozoa in eastern Canadian waters with special reference to tintinnid feeding, nitrogen excretion and reproduction rates. Ph.D. Thesis, Dalhousie Univ. 154 pp.

Kamptner, E. 1927. Beitrag zur Kenntnis adriatischer coccolithophoriden. *Arch. F. Protistenk.* **58:** 173–184.

Lohmann, H. 1902. Die coccolithophoridae usw. *Arch. F. Protistenk.* **1:** 89–165.

Needler, A. B. 1949. Paralytic shellfish poisoning and *Gonyaulax tamarensis. J. Fish Res. Bd. Can.* **7:** 490–504.

Paranjape, M. A. 1980. Occurrence and significance of resting cysts in a hyaline tintinnid, *Helicostomella subulata* (Ehre.) Jorgensen. *J. Exp. Mar. Biol. Ecol.* **48:** 23–34.

Prakash, A. 1963. Source of paralytic shellfish toxin in the Bay of Fundy. *J. Fish Res. Bd. Can.* **20:** 983–996.

Rassoulzadegan, F. 1978. Dimensions et taux d'ingestion des particules comosommees par un tintinnide: *Favella ehrenbergii* (Clap. et Lachm.) Jorg., Cilie pelagique marin. *Ann. Inst. Oceanogr. Paris* **54:** 17–24.

Reid, P. C., and A. W. G. John. 1978. Tintinnid cysts. *J. Mar. Biol. Assoc. U. K.* **58:** 551–557.

Sokal, R. R., and F. J. Rohlf. 1969. *Biometry.* W. H. Freeman and Co., San Francisco. 776 pp.

Spittler, P. 1973. Feeding experiments with tintinnids. *Oikos Supplementum,* **15:** 128–132.

Taylor, D. L. 1969. Identity of zooanthellae isolated from some Pacific Tridacnidae. *J. Phycol.* **5:** 336–340.

Taylor, D. L. 1971. Taxonomy of some common *Amphidinium* species. *Br. Phycol. J.* **6:** 129–133.

Taylor, R. D., M. Ikawa, J. J. Sasner, Jr., F. P. Thurburg, and K. K. Anderson. 1974. Occurrence of choline esters in the marine dinoflagellate *Amphidinum carteri. J. Phycol.* **10:** 279–283.

Thurburg, F. P., and J. J. Sasner, Jr. 1973. Biological activity of a cell extract from the dinoflagellate *Amphidinum carteri. Chesapeake Sci.* **14:** 48–69.

Wangersky, P. J., and R. R. L. Guillard. 1960. Low molecular weight organic base from the dinoflagellate *Amphidinum carteri. Nature* **185:** 689–690.

White, A. W. 1979. Dinoflagellate toxins in phytoplankton and zooplankton fractions during a bloom of *Gonyaulax excavata.* Pp. 381–384 in D. L. Taylor and H. H. Seliger, Eds., *Toxic dinoflagellate blooms.* Elsevier North Holland, Inc., New York.

Reference: *Biol. Bull.*, **160:** 146–154. (February, 1981)

THE LIFE HISTORY, DEVELOPMENTAL STAGES, AND TAXONOMIC RELATIONS OF THE DIGENETIC TREMATODE *LASIOTOCUS MINUTUS* (MANTER, 1931) THOMAS, 1959[1]

HORACE W. STUNKARD

The American Museum of Natural History, Central Park West at 79th Street, New York, N. Y. 10024; and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Abstract

Cercaria adranocerca n. sp. was described by Stunkard and Uzmann (1959) from the clam *Gemma gemma* at Boothbay Harbor, Maine. Further study of the species at Woods Hole, Massachusetts, has afforded information for revision and correction of the original description. The cercariae are microcercous and after emergence from their sporocysts are encysted in the haemocoele of the clam. They may be extruded, either singly or embedded in a jelly-like matrix, and float in seawater. Feeding metacercariae to *Menidia menidia* yielded developmental stages to mature worms, identified as *Lasiotocus minutus* (Manter, 1931) Thomas, 1959. Discovery of the life cycle may aid in resolution of the controversy concerning the status of the genera *Lasiotocus* Looss, 1907; *Genolopa* Linton, 1910; and *Proctotrema* Odhner, 1911.

Introduction

Lasiotocus minutus, a parasite in the intestine of *Menidia menidia,* was described and named by Manter (1931) from specimens taken at Beaufort, North Carolina. It is a member of the Monorchiidae, a large family with 12 subfamilies, about 30 genera, and 100 species, which infect marine fishes in all parts of the world. Observations on different stages in the life history of certain species have been recorded and the complete life cycle of a single species, *Monorcheides cumingiae,* was worked out and published by Martin (1940).

In a paper on the life history of *Proctoeces maculatus,* Stunkard and Uzmann (1959) included the description of a microcercous cercaria produced in sporocysts in the small bivalve *Gemma gemma.* The clams were taken in August and September, 1957, in the region of Boothbay Harbor, Maine. The asexual generations were described under the designation, *Cercaria adranocerca,* and type specimens were deposited in the U. S. National Museum under the number 56236. Although the excretory vesicle was saccate rather than Y-shaped, the cercariae were microcercous and otherwise similar to those of *Proctoeces maculatus*; accordingly, they were assigned tentatively to the family Fellodistomidae. The species was found in *G. gemma* of the Woods Hole region and further study has shown it to be the larval stage of *Lasiotocus minutus.*

Material and Methods

Between 1 June and 1 August 1980 more than 4000 specimens of *G. gemma* were collected from different locations in the area of Woods Hole. Infection with *C. adranocerca* varied from 2 to 5%. Most of the clams were maintained in small

---

Received 18 August 1980, accepted 7 November 1980.

[1] Investigation supported by NSF-DEB-80-06150.

bowls, 25 to 50 in each bowl, to obtain metacercariae. With a set of 25 bowls, metacercariae were available almost every day. Completion of the life cycle proved *Cercaria adranocerca* to be the larval stage of *Lasiotocus minutus.*

Dissection of clams provided the asexual generations of the species. There are at least two generations of sporocysts (Figs. 1, 2) with individuals of varying sizes in the haemal sinuses of *G. gemma.* They invade both the digestive gland and the gonad. Small individuals contain a few germ-balls and incipient cercariae while large ones, filled with developing cercariae, reach 1.20 mm in length and 0.20 mm in diameter. The birth pore is terminal and this end may be protruded. Small specimens are motile, but large, gravid sporocysts are incapable of movement.

The cercariae are microcercous, without stylets or ocelli, and are not liberated from the mollusk. Rather, they are encysted while in the haemocoele of the clam. Here, they may aggregate in clusters (Fig. 3), or may be extruded either singly or in strands (Fig. 4) embedded in a jelly-like matrix. When liberated in the sea, the metacercariae, either individually or in clusters, float and are carried about by water currents. Eventually they sink to the bottom and may be ingested by small crustaceans or other carnivorous invertebrates. When discharged in strands, each mass may contain as many as 500 metacercariae (Fig. 4). The emergence of metacercariae apparently is not determined by either light or temperature. They are found early in the morning and late in the afternoon. Floating strands of metacercariae, often embedded in bits of clam or other material, were fed to small fishes: *Menidia menidia, Fundulus heteroclitus,* and flatfishes (0-year *Pseudopleuronectes americanus*). The fishes were collected in adjacent waters and were naturally infected. At Woods Hole, *M. menidia* harbors two species of *Lasiotocus*: *L. minutus* (Manter, 1931) Thomas, 1959 and *L. elongatus* (Manter, 1931) Thomas, 1959 (described in Stunkard, 1981). Developmental stages of the two species may overlap in size but may be distinguished by specific characters and especially by the sizes of suckers. In *L. minutus,* the oral sucker is slightly larger than the acetabulum, whereas in *L. elongatus* the acetabulum is the larger. For experimental infections, the fishes were isolated for about 2 weeks and fed on living tissues that did not contain parasites. Then, at intervals of about 1 week, they were fed metacercariae shed by *G. gemma.* Large strands were added to the aquaria and pieces were embedded in bits of clam or other tissue that insured that they would be eaten. Dissection of fishes at intervals yielded all stages of development of *L. minutus,* from metacercariae to gravid adults, and established that *C. adranocerca* is the larva of that species. The infection of fishes occurs without the intervention of a second intermediate host, but the metacercariae may be eaten by small crustaceans and their ingestion by *M. menidia* is not precluded. No infection was established in either *F. heteroclitus* or *P. americanus.*

DESCRIPTIONS

Sporocysts

Stunkard and Uzmann (1959) reported that in *C. adranocerca* the sporocysts (Figs. 1, 2) were relatively few and oval to sausage-shaped; the largest 0.42 mm long and 0.11 mm wide; and smaller ones, no larger than a cercaria, containing a few germ balls. Study of more abundant material permitted additions to the description. The number of sporocysts was small, but gravid specimens, more than 1 mm long and 0.13 mm wide, were commonly present and filled with developing cercariae. Since there are successive generations of sporocysts, it is apparent that the cercariae are produced in daughter sporocysts. The daughters are recognizable when very small and may be distinguished from cercariae by the formation of an internal lumen, containing a few morula-like clusters of more deeply staining cells. As development proceeds, it appears that the progeny consists of a few daughters and then the sporocysts produce only cercariae.

Cercariae

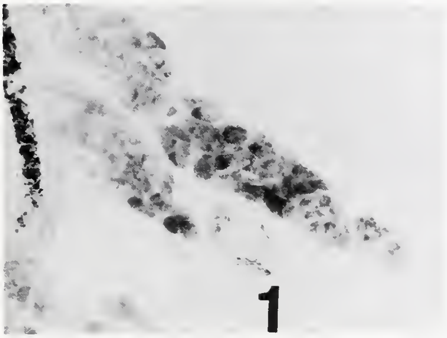
A representative account of the cercaria (Figs. 2, 7) was given by Stunkard and Uzmann (1959). The data were taken from specimens found on dissection of two clams and it is now evident that those infections were recent, not fully mature. The measurements were smaller than those obtained in the study of specimens in older and well established infections. The earlier account reported cephalic-gland ducts in the region of the oral sucker. That observation has not been confirmed. From the level of the pharynx to the posterior ends of the caeca, cystogenous glands underlie the body wall, except for the areas occupied by the excretory vesicle and reproductive organs. Extrusion of secretion from these glands in the oral area probably was misconstrued in the earlier report. Since the cercariae do not invade a second intermediate host, there is no reason for penetration glands.

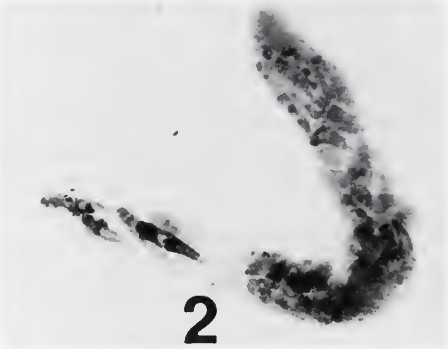
Heat-fixed cercariae had the following average measurements: length, 0.17 mm; width, 0.052 mm; acetabulum, 0.025 mm; oral sucker, 0.030 mm; pharynx, 0.014 mm. The tail, 0.006 mm in diameter, is attached ventrally. There are two lateral excretory pores at the body-tail junction. The excretory vesicle was described as saccate, but as it is filled with fluid and spherical concretions, it may extend forward to the acetabulum. In this condition (*i.e.*, when extended) the collecting ducts open into the vesicle some distance posterior to its anterior end.

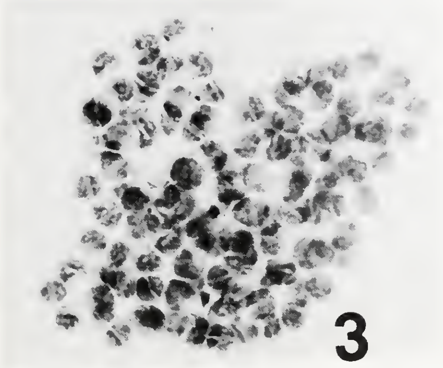
The 1959 report tentatively assigned *Cercaria adranocerca* to the family Fellodistomidae. Further information has demonstrated that the species is a member of the family Monorchiidae. The earlier error is explained by the report of Martin (1940) that in *Monorcheides cumingiae,* the only marine species whose life cycle was then known, the cercaria is biocellate and has a long tail with lateral cup-shaped lappets. However, Cable (1956) reported monorchiid cercariae from Puerto Rico, one species with eye-spots and a long tail like *M. cumingiae* and another without ocelli and with the tail reduced to a tiny knob. His (1965) review of the subject of cercarial tails submitted evidence that tails may be deceptive and misleading: The same type may be present in different families and different types in the same family.

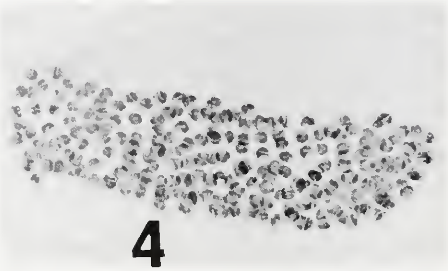
Metacercariae

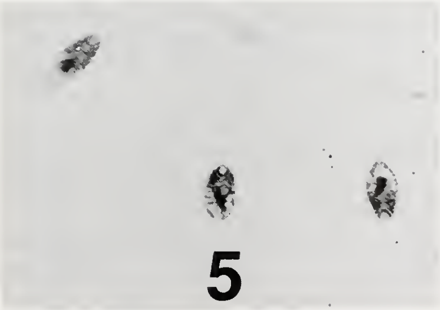
Existence as cercariae is brief and transitory. Soon after it emerges from the sporocyst, each cercaria begins a series of writhing movements that cause the emission of the secretion from the cystogenous glands. This colorless, transparent material forms a large, loose, flexible, membranous sac around the metacercaria (Figs. 3, 4). The distal tip of the sac, especially, is adhesive. When infection is heavy the metacercariae may form clusters (Fig. 4). When the number of the cercariae is small, they may be extruded singly, or two or more may attach to a bit of debris or to each other (Fig. 8). In such cases, on fixation, the walls of the sacs collapse, forming filaments 0.15 to 0.18 mm long, connecting the metacercariae to the object or to each other. The metacercariae, fixed, stained and mounted, measure 0.07–0.08 mm in diameter. Floating strands of living metacercariae (Fig. 9) may measure 5.00 mm in length and 0.90 mm in width. It appears that the size and shape of the strand is determined by the diameter of the siphon.


1


2


3


4


5

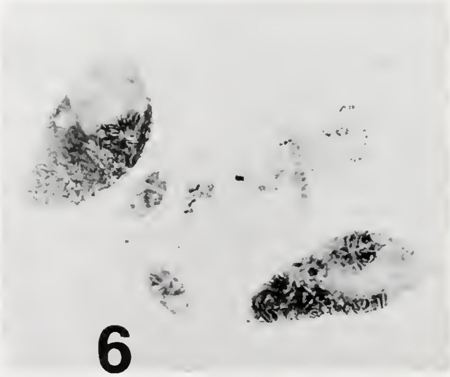

6

FIGURE 1. Ends of sporocysts in tissue of *G. gemma*, 0.16 mm in diameter. The black material is in the rectum of the clam.

FIGURE 2. Isolatec sporocyst with germ-balls and cercariae; anterior and extended. Total length 1.16 mm.

FIGURE 3. A cluster of encysted cercariae in the mantle cavity of the clam, each 0.045 mm in diameter, alive, and active.

FIGURE 4. A strand of metacercariae after extrusion from *G. gemma*, embedded in matrix, floating in bowl, alive; 5.7 × 0.94 mm.

FIGURE 5. Juvenile specimens, 10 days development in *M. menidia*, fixed, stained and mounted; specimens 0.115 × 0.046 mm.

FIGURE 6. Mature and juvenile specimens, natural and experimental infections in the same fish, fixed, stained and mounted; mature specimens, 0.325 × 0.130 mm; juveniles, 0.09 × 0.05 mm.

Adults

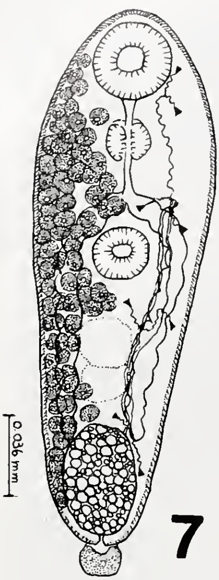
The adult worms (Figs. 11, 12) recovered from *M. menidia* were identified as *Lasiotocus minutus.* Gravid specimens measured 0.30–0.70 mm in length and 0.10–0.25 mm in width. They are almost cylindrical and taper toward both ends, although more rounded anteriorly. The integument bears closely set, flattened spines disposed in quincuncial arrangement. They diminish in size and number posteriorly. The body wall is composed of the usual circular, longitudinal and diagonal muscular layers; it is strong and well developed for such a small worm. The acetabulum shifts forward as the reproductive organs mature, from near midbody to the anterior third. It is somewhat smaller than the oral sucker, 0.042–0.050 mm in diameter. Both suckers appear larger when the lumen is expanded and the opening is enlarged. The oral sucker is subterminal, 0.052–0.058 mm in diameter, and the mouth is ventral. The short prepharynx and somewhat longer esophagus may both disappear when the anterior end of the worm is retracted. The pharynx is 0.020–0.025 mm in diameter; the digestive tract bifurcates a short distance anterior to the acetabulum and the caeca terminate near the anterior end of the excretory vesicle when it is saccate and relatively empty. The genital pore is located immediately anterior and somewhat lateral to the acetabulum and opens from a common genital atrium. The single testis, typically oval, 0.05–0.07 mm in diameter, is located near midbody but shifts anteriorly as the posttesticular region fills with uterine coils. The cirrus sac is clavate; it extends over and posterior to the acetabulum and under coverglass pressure may be displaced to the right or left. The sac contains a large, oval seminal vesicle followed by the prostate and the ejaculatory duct, which may be protruded as a cirrus. The duct is lined with small triangular spines 0.004–0.005 mm in length with curved tips. The ovary is spherical to oval, 0.025–0.030 mm in diameter, situated anterior or ventral to the anterior part of the testis. The oviduct arises from the posterior end and is joined by the duct from the seminal receptacle, which continues, as Laurer's canal, to open on the dorsal side of the body. The oviduct then receives the common vitelline duct and enters Mehlis' gland to become the ootype. It is continued by the initial part of the uterus. The proximal part of the uterus contains spermatozoa and continues as a much coiled series of loops that fill the posterior half of the body, covering the gonads and the lateral areas of the body to the level of the pharynx. The metraterm is short, bearing conical spines 0.005–0.006 mm in length with rounded bases. It enters the genital atrium ventral and posterior to the opening of the ejaculatory duct. Eggs in the initial portion of the uterus have thin, transparent shells, and the embryos stain intensely; eggs in the distal coils have thick yellow shells and measure 0.020–0.022 mm in length and 0.012–0.013 mm in width. The eggs are operculate, with an opening 0.008–0.009 mm in diameter. They are embryonated when passed, but the miracidia do not emerge when maintained in seawater. The egg shells are firm, but under coverglass pressure they may flatten and appear slightly larger. The vitellaria consist of dorsal follicles, and lateral to the ovary. Vitelline ducts pass mediad on each side and unite to form a vitelline receptacle that discharges into the oviduct just before it enters Mehlis' gland. A few vitelline cells and a single ovum are incorporated into each egg in the gland. The description of the excretory system, vesicle, tubules, and flame-cells is in the original account (Stunkard and Uzmann, 1959). The vesicle was described as saccate, but it may extend forward toward the acetabulum as it fills with fluid and spherical concretions. In this condition, the collecting ducts open into the vesicle some distance from its anterior end.

The worms agree substantially with the diagnosis of the species as given by Manter (1931), but not with his figure 2 of the species, in which the ovary and vitellaria are some distance posterior to the acetabulum. Also, it is obvious that the egg size given by Manter is for collapsed, not normal, eggs. Manter (1931) listed *Monostomum* sp. Linton 1905, p. 356, from *Menidia menidia* and *Fundulus majalis* at Beaufort, North Carolina as a synonym of *Genolopa minuta.* Actually, that species is a synonym of *Lasiotocus elongatus,* not *Lasiotocus minutus.*

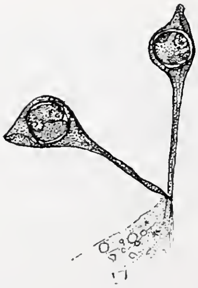
DISCUSSION

The integrity and validity of the genera *Lasiotocus* Looss, 1907; *Genolopa* Linton, 1910; and *Proctotrema* Odhner, 1911, have long been in dispute. *Lasiotocus* was based on *Distoma mulli* (Stossich, 1883) from *Mullus barbatus* taken at Trieste in the Adriatic. *Genolopa* was based on *Genolopa ampullacea* n. sp., from *Haemulon* spp., and other fishes at Tortugas, Florida, and included other specimens described and figured as *Monostomum* sp. in Linton (1907), from the same host-fishes taken in Bermuda. *Proctotrema* was based on *Proctotrema bacilliovatum* n. sp., from *Mullus barbatus* taken at Trieste. The worms were from the same host-species and location as *Lasiotocus mulli* (Stossich, 1883) and their relations were considered. Odhner (1911, p. 250) stated, "Hieraus geht indessen hervor, dass sich diese Form unter dem vom Verfasser eingesammelten Triester Materiale befindet und von ihm schon untersucht worden ist; unter solchen Umständen möchte ich den Mitteilungen des älteren Kollegen nicht vorausgreifen, sondern begnüge mich damit festzustellen dass *Lasiotrema mulli* mit *Proctotrema bacilliovatum* nachstverwandt ist." He described the eggs as 31–33 by 8–9 μm and reported, p. 250, "sie legen sich mit parallel Langsachsen in kleinen Bündeln zusammen." This statement is a misrepresentation, from collapsed, distorted eggs. Linton's paper was not available to Odhner.

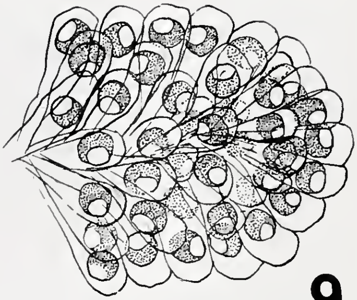
Synonymy of *Genolopa* and *Proctotrema* has been discussed by many authors. Lloyd and Guberlet (1932) declared that information was too inadequate to warrant a decision. Yamaguti (1934) predicated the probable identity of *Genolopa* and *Proctotrema,* since it was apparent that Linton (1910) had overlooked the acetabulum in *Genolopa.* Indeed, Linton did not mention the acetabulum; he described a genital sucker, actually the genital atrium, located immediately anterior to the acetabulum. Manter (1940) distinguished between *Genolopa* and *Proctotrema* by differences in spination of the copulatory organs. He suggested that *Genolopa* be restricted to species with a median cluster of longer spines in the cirrus sac, a feature of *G. ampullacea.* Referring to *Genolopa,* Hopkins (1941) declared, p. 399, "the type specimen agrees with Odhner's (1911) description of the genus *Proctotrema* in every point except for the more rounded shape of the oral sucker, the non-lobate form of the ovary (which may be a result of flattening, since the ovary in other species with tri-lobate ovary is more rounded in flattened specimens), and the less elongate shape of the eggs in *G. ampullacea.* Since these are minor differences, and since other species completely cover the range of differences in even these characteristics, it is obvious that *Proctotrema* Odhner, 1911, is a synonym of *Genolopa* Linton, 1910. In spite of Manter's statement, the spination of the cirrus in *G. ampullacea* is in no way exceptional, and there is no 'median cluster of much longer spines in the cirrus sac' in the type specimen." Hopkins transferred all described species from *Proctotrema* to *Genolopa.* Manter (1942) described nine species of monorchiid trematodes from fishes at Tortugas, Florida. He gave a



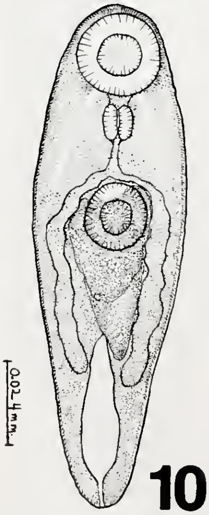
7



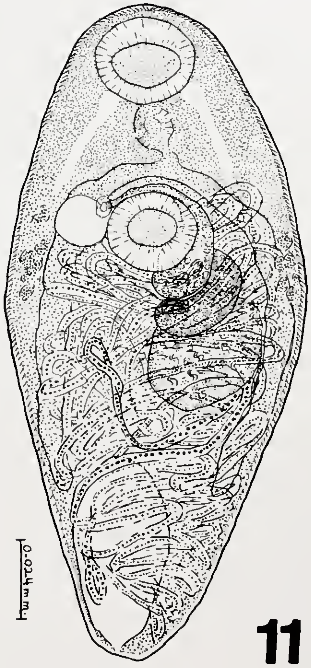
8



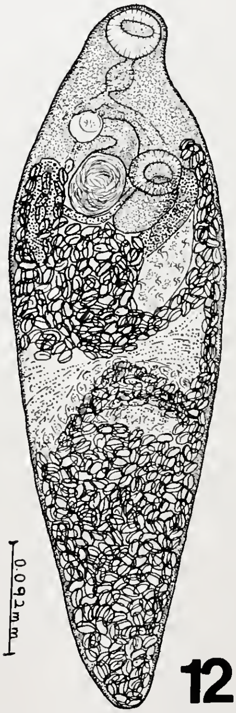
9



10



11



12

FIGURE 7. Cercaria, drawn from pencil sketches; cystogenous gland cells on one side, excretory system on the other side.

FIGURE 8. Isolated metacercariae attached by filaments to a bit of debris; fixed, stained and mounted.

FIGURE 9. End of a strand of floating metacercariae embedded in matrix, drawn from a living specimen.

FIGURE 10. Juvenile specimen from *M. menidia*, experimental infection; fixed, stained and mounted, 0.15 mm long.

FIGURE 11. Mature but not fully gravid specimen (no shelled eggs) fixed, stained and mounted; length 0.196 mm. Drawn to the same scale as Figure 10.

FIGURE 12. Fully gravid specimen, fixed, stained and mounted, length, 0.68 mm.

detailed description of *G. ampullacea,* formulated a new diagnosis of *Genolopa,* and insisted on the validity of *Proctotrema.* Manter and Pritchard (1961) reported on digenetic trematodes of Hawaiian fishes. They accepted the validity of *Lasiotocus, Genolopa,* and *Proctotrema,* but added, p. 483, "The taxonomy of the family Monorchiidae is in a state of considerable uncertainty."

The status of *Lasiotocus* remains equivocal. Dollfus (1948) gave a detailed description of *L. mulli,* with figures of an entire specimen and of the copulatory organs. Thomas (1959) described five species of monorchiid species from fishes taken on the coast of Ghana. He reviewed the family Monorchiidae, gave a revised diagnosis of *Lasiotocus,* and suppressed *Proctotrema* as a synonym of *Lasiotocus.* Accordingly, he transferred some 16 species, including *Genolopa minuta* Manter, 1931, to *Lasiotocus.* Bartoli and Prévot (1966) gave a new description of *Proctotrema bacilliovatum* Odhner, 1911, the first study of the species since Odhner's 1911 original description. The specimens came from the rectum of *Mullus barbatus* at Trieste. The description by Odhner was brief and inadequate; the account by Bartoli and Prévot detailed and well illustrated. They reported that *P. bacilliovatum* appeared to be distinctive, but all species previously assigned to *Proctotrema,* except *P. bacilliovatum,* were transferred to *Lasiotocus.*

In his *Synopsis of digenetic trematodes of vertebrates,* Yamaguti (1971) listed 12 subfamilies in the family Monorchiidae but the family diagnosis was so diverse that its unity was impaired. The subfamily Lasiotocinae contained 20 genera, including *Lasiotocus, Genolopa* and *Proctotrema.* Data for the precise systematic determination of these trematodes is still inadequate. Knowledge of their asexual generations and intermediate hosts may help resolve the situation.

LITERATURE CITED

Bartoli, P., and G. Prévot. 1966. Contribution à l'étude des Monorchiidae (T. Odhner) parasites de poissons du genre *Mullus* en Méditerranée. Description de *Timonia mediterranea* n. gen., n. sp. (Trematoda-Digenea) *Ann. Parasitol.* **41:** 397–412.

Cable, R. M. 1956. Marine cercariae of Puerto Rico. Sci. Surv. Puerto Rico and Virgin Islands. *N. Y. Acad. Sci.* **16:** 491–577.

Cable, R. M. 1965. Thereby hangs a tail. *J. Parasitol.* **51:** 3–12.

Dollfus, R. Ph. 1948. L'énigme de *Distoma furcatum* Bremser expliquée. Contribution à la connaissance des trématodes des poissons du genre *Mullus* en Méditerranée. *Bull. Instit. Oceanogr.,* No. **928.** 23 pp.

Hopkins, S. H. 1941. New genera and species of the family Monorchiidae (Trematoda) with a discussion of the excretory system. *J. Parasitol.* **27:** 395–407.

Linton, E. 1907. Parasites of Bermuda fishes. *Proc. U. S. Nat. Museum* **33:** 85–126.

Linton, E. 1910. Pp. 11–98 in *Helminth fauna of the Dry Tortugas. II. Trematodes.* Publ. No. 133, Carnegie Instit. Washington, D. C.

Lloyd, L. C., and J. E. Guberlet. 1932. A new genus and species of Monorchiidae. *J. Parasitol.* **18:** 232–239.

Manter, H. W. 1931. Some digenetic trematodes of marine fishes of Beaufort, North Carolina. *Parasitology* **23:** 396–411.

Manter, H. W. 1940. Digenetic trematodes of fishes from the Galapagos Islands and the neighboring Pacific. *Allan Hancock Pacific Exped.* **2:** 329–497.

Manter, H. W. 1942. Monorchiidae (Trematoda) from fishes of Tortugas, Florida. *Trans. Amer. Microsc. Soc.* **61:** 349–360.

Manter, H. W. and M. H. Pritchard. 1961. Studies on digenetic trematodes of Hawaiian fishes: Family Monorchiidae and Haplosplanchnidae. *J. Parasitol.* **47:** 483–492.

Martin, W. E. 1940. Studies on the trematodes of Woods Hole. III. The life-cycle of *Monorcheides cumingiae* (Martin) with special reference to its effect on the invertebrate host. *Biol. Bull.* **79:** 131–144.

Odhner, T. 1911. Zum natürlichen System der digenen Trematoden. II. *Zool. Anz.* **37:** 238–253.

Stunkard, H. W. 1981. The morphology, life history, and systematic relations of *Lasiotocus elongatus* (Manter, 1931) Thomas, 1959 (Trematoda-Digenea) *Biol. Bull.* **160:** 155–160.

Stunkard, H. W., and J. R. Uzmann. 1959. The life-cycle of the digenetic trematode, *Proctoeces maculatus* (Looss, 1901) Odhner, 1911 [(syn. *P. subtenuis* Linton, 1906) Hanson, 1950], and description of *Cercaria adranocerca* n. sp. *Biol. Bull.* **116:** 184–193.

Thomas, J. D. 1959. Trematodes of Ghanian sub-littoral fishes. I. The family Monorchiidae. *J. Parasitol.* **45:** 95–112.

Yamaguti, S. 1934. Studies on the helminth fauna of Japan. Pt. 2. Trematodes of fishes, II. *Jap. J. Zool.* **5:** 249–541.

Yamaguti, S. *Synopsis of digenetic trematodes of vertebrates.* Vol. I, 1074 pp; vol. II, 349 Plates, 1796 Figs. Keigaku Publishing Co., Tokyo.

Reference: *Biol. Bull.*, **160:** 155–160. (February, 1981)

THE MORPHOLOGY, LIFE HISTORY, AND SYSTEMATIC RELATIONS OF *LASIOTOCUS ELONGATUS* (MANTER, 1931) THOMAS, 1959 (TREMATODA: DIGENEA)[1]

HORACE W. STUNKARD

The American Museum of Natural History, Central Park West at 79th Street, New York, N. Y. 10024; and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT AND INTRODUCTION

Throughout its range, *Menidia menidia* harbors two species of *Lasiotocus: L. minutus* (Manter, 1931) and *L. elongatus* (Manter, 1931). The life cycle of *L. minutus* was described by Stunkard (1981). That of *L. elongatus* is recorded in the present report. The asexual generations of both species occur in the bivalve, *Gemma gemma.* Developmental stages of the two species overlap in size, but may be distinguished by specific characters, especially the relative sizes of the suckers and the length of the digestive caeca. In *L. minutus* the excretory bladder contains concretions, but none was observed in *L. elongatus.*

Lasiotocus elongatus was described by Manter (1931) and named *Genolopa elongata* n. sp. It is a common parasite of *M. menidia* along the Atlantic coast of North America. The type specimens were taken in 1928 in a collection of trematodes from marine fishes made at Beaufort, North Carolina. The description was brief, consisting of 14 lines of text and a single figure (Fig. 3).

Actually, the species was discovered by Linton at Woods Hole, Massachusetts. In his list of the "parasites of *Menidia menidia*, Silversides," he reported six specimens taken on 28 August 1899 and two on 30 August 1899. The description in Linton (1901, p. 444) reads, "*Distomum* sp. Small, short, fusiform. (Pl. XXXII, Figs. 357, 358). Resembling *D. bothryophoron* Olsson, but with more slender neck and distinct esophagus." The figures are so characteristic there is no doubt of their identity. Manter (1931) did not refer to Linton's (1901) report. Linton (1905) recorded the species from *M. menidia* at Beaufort, North Carolina. The report states, p. 360, "*Distomum* sp. This form resembles that figured in Parasites of Fishes of the Woods Hole Region, Figs. 357, 358. The body of this worm is densely clothed with extremely minute spines." Measurements were given for two specimens. The description, except for egg size, which obviously is not correct, is adequate for specific recognition, as admitted by Manter (1931), who assigned the species to *Genolopa minuta.* Linton reported on the stomach contents of the fishes he examined, and this information is of primary significance for students attempting to discover the intermediate hosts and bionomics of the parasites he described.

Although *L. elongatus* has been listed by certain authors, and transferred from *Genolopa* Linton, 1910, to *Paraprocototrema* Yamaguti, 1934, by Sobolev (1955) and to *Lasiotocus* Looss, 1907, by Thomas (1959), there has been no description since the original account by Manter. The discovery of the molluscan host and description of the asexual generations provide information for determining systematic and taxonomic relations. The validity and status of the genera *Lasiotocus* Looss, 1907; *Genolopa* Linton, 1910; and *Proctotrema* Odhner, 1911, are uncertain and controversial.

Received 9 October 1980, accepted 7 November 1980.

[1] Investigation supported by NSF-DEB-80-06150.

MATERIALS AND METHODS

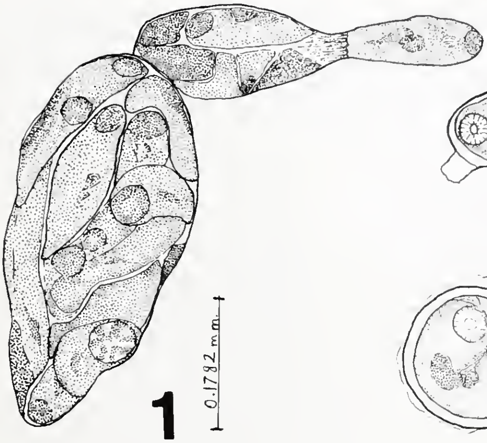
Dissection of *Gemma gemma* at Boothbay Harbor, Maine, in 1957 disclosed infection by a microcercous cercaria, described by Stunkard and Uzmann (1959) as *Cercaria adranocerca* n. sp. Study of *G. gemma* was resumed at Woods Hole, in an attempt to find *C. adranocerca* and discover the life cycle of the species. According to Abbott (1974), *G. gemma* occurs from Nova Scotia to Florida, Texas, and the Bahamas. In the summer of 1980, over 5000 specimens of *G. gemma* were collected from various locations in the Woods Hole area. The clams were maintained in small bowls. No cercariae were shed, but encysted metacercariae were liberated into the seawater. Two types of metacercariae were recognized and, accordingly, the clams shedding each type were identified by isolation. Type II metacercariae were liberated as individuals, each in a large, flexible, membranous sac, or in strands composed of hundreds of individuals embedded in a jelly-like matrix. They were suspended and floated in seawater. The sporocysts and cercariae were obtained by dissecting the infected clams, and proved identical to those described as *C. adranocerca*. When fed to *Menidia menidia*, these metacercariae developed to adults identified as *Lasiotocus minutus* (Manter, 1931). Descriptions of the successive stages in the life-cycle and taxonomic relations of the species are in Stunkard (1981).

Type I metacercariae were liberated singly, each in a thick-walled cyst, partially enclosed in an unformed, narrow coat of jelly-like material. Incidence of infection was less than 1.00%. These cysts sank in seawater and adhered lightly to algae and other objects in the water. They were fed to small fishes—*Menidia menidia, Fundulus heteroclitus*, and *Pseudopleuronectes americanus*—collected in the Woods Hole area and all naturally infected when taken. The fishes were isolated for 2 or 3 weeks, during which time they were fed bits of tissue that did not contain parasites and the trematodes of natural infection matured. After this preliminary period, they were fed each week with Type I cysts, embedded in bits of clam or squid. Dissection of these fishes at weekly intervals yielded a consecutive series of developmental stages from recently excysted metacercariae to sexually mature specimens, identified as *Lasiotocus elongatus*. No infections were established in *F. heteroclitus* or *P. americanus*.

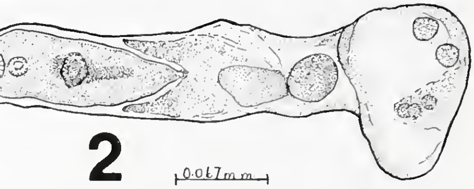
DESCRIPTIONS

Sporocysts

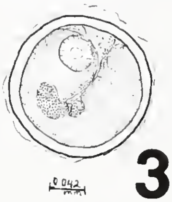
Sporocysts (Figs. 1, 2) occurred in the haemal sinuses of the digestive gland and sometimes in the gonad of *G. gemma*. They were oval to elongate, typically with an oval basal portion and an anterior portion sometimes extended to a tapering tip that bears the birth pore. There were successive generations of sporocysts; cercariae were produced in daughters after they had ceased forming sporocysts. Young sporocysts tended to be smaller than cercariae and could be recognized by the presence of a lumen containing a few germ balls. The sporocysts varied in shape and size; the largest (Fig. 1) was 0.94 mm long and 0.22 mm in greatest width. There was little or no motility in large sporocysts, but mature cercariae in them sometimes were very active. The cercariae were derived from deeply staining germinal cells embedded in the mesenchymal layer under the membranous tegument of the sporocyst. These cells cleaved to form germ-balls, cercarial embryos liberated into the lumen of the sporocyst. Figure 2 shows all stages in a young sporocyst, 0.33 mm long and 0.040 mm wide. The basal portion contains individual, heavily



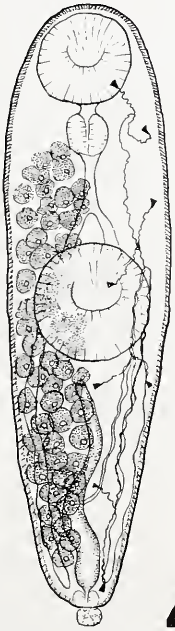
1



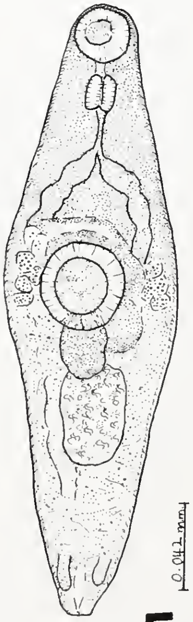
2



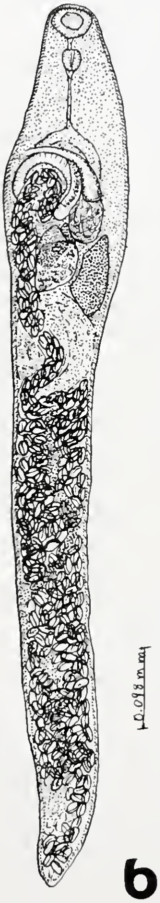
3



4



5



6

FIGURE 1. Mature daughter sporocyst, 0.94 mm long, with a cercaria emerging through the birth pore.

FIGURE 2. Young daughter sporocyst, 0.33 mm long and 0.040 mm wide, with all developmental stages from isolated germinal cells to germ-balls of increasing size and a fully formed cercaria.

FIGURE 3. Encysted metacercaria, recovered from seawater, 0.072 mm in diameter.

FIGURE 4. Cercaria, drawn from pencil sketches of living specimens, showing cystogenous glands, and details of the digestive and excretory systems.

FIGURE 5. Juvenile specimen, becoming sexually mature, 0.32 mm long.

FIGURE 6. Mature, gravid specimen, 0.90 mm long.

staining germinal cells. In addition there are small morulae of cleaving cells and two small germ-balls, each 0.014 mm in diameter and apparently consisting of 32 cells. The remainder of the specimen contains a larger germ-ball, 0.035 by 0.028 mm; two later stages; and a fully formed cercaria, 0.14 mm long and 0.042 mm wide.

Cercariae

As noted, the cercariae (Fig. 4) were not liberated from the mollusk. Cercariae were obtained only by dissection of clams, and the number present was always small. Ordinarily, they encysted almost immediately after emergence from the sporocyst. If immature cercariae were released, they sometimes encysted, and such metacercariae were smaller than normal. Differences in size of cercariae and metacercariae were the result of premature emergence. Fully developed cercariae were elongate, fusiform, widest at or near the acetabular level, and more attenuated posteriorly. They were active and different shapes were produced by the contractions of muscles in the body wall. Although the shape was not specific and location of organs shifted as different shapes were assumed, the conformation was consistent and always recognizable. In fixed specimens the sizes of the suckers were definite, but in living worms the sizes were not constant; when expanded with wide openings they appeared much larger than when contracted. The body-wall was well developed; strong for so small a worm; and composed of the usual circular, longitudinal, and diagonal muscle layers. The integument contains large, closely set spines, as noted by Linton (1905). The body wall was underlaid with cystogenous cells, arranged in longitudinal rows that were conspicuous in the anterior part of the body and may simulate penetration glands. Measurements of heat killed, fixed, stained, and mounted specimens gave the best morphological data. They varied from 0.18 to 0.21 mm in length, and 0.053 to 0.060 mm in width. The acetabulum, situated near midbody, was 0.030 to 0.038 mm in diameter; the oral sucker 0.027 to 0.032 mm in diameter; the prepharynx, 0.005 to 0.012; the pharynx, 0.015 to 0.020 mm in diameter; the esophagus, 0.010 to 0.020 mm in length. The caeca were long, extending to the posterior end of the body. The reproductive organs were represented by a narrow column of germinal cells extending from the level of the acetabulum about one-third to one-half the distance from the acetabulum to the posterior end of the body. The excretory vesicle was saccate to tubular and opened to the surface through two lateral pores, situated at the body-tail junction. It was closed at the posterior end by a strong sphincter. The vesicle was lined with epithelial cells and when filled sometimes extended to the level of the acetabulum. Collecting ducts opened into it on either side, near the anterior end when it was empty and farther posterior as it filled with fluid. No excretory concretions were observed. The ducts led forward to the level of the acetabulum and divided to form anterior and posterior secondaries. Each secondary divided into tertiaries, and the pattern and location of the flame cells are depicted in Figure 4. The flame-cell formula is $[(2 + 2) + (2 + 2)]$, typical for the subfamily Lasiotocinae.

Metacercariae.

Encysted, the metacercariae (Fig. 3) were bent, with the dorsal surface in contact with the cyst wall, and the ventral surface located internally. Fixed, stained, and mounted specimens were 0.070 to 0.075 mm in diameter. The wall was clear, 0.003 mm thick.

Juveniles and Adults

Recently excysted juvenile specimens were not much larger than cercariae, but the cystogenous glands had disappeared and there had been development of the reproductive organs. Figure 5 portrays a specimen 0.32 mm in length and 0.071 mm broad. The acetabulum is 0.034 mm in diameter, the oral sucker, 0.031 mm; the prepharynx is 0.011 mm long, the pharynx is 0.021 by 0.018 mm; and the esophagus is 0.022 mm long. The gonads are functional. The initial loops of the uterus contain spherical to oval eggs, with thin, membranous, flexible shells and deeply staining miracidial embryos. Uterine loops obscure the digestive caeca in the region posterior to the testis.

Among adults (Fig. 6), gravid specimens, fixed, stained, and mounted, were 0.40 to 1.20 mm in length, 0.07 to 0.16 mm in width. The body was fusiform, widest at the level of the acetabulum, tapering toward the ends. The anterior end, especially, extended and retracted, with the shape of the body modified accordingly. The worms were almost cylindrical and living specimens easily rolled over under a coverslip. The posterior half of the body was filled with uterine loops that overlapped and obscured other structures, especially the intestinal caeca and excretory vesicle. The body wall comprised the usual circular, longitudinal, and oblique muscles but was not strongly developed. The acetabulum was in the anterior fourth of the body and was 0.065 to 0.070 mm in diameter. Over the entire surface, the integument bore closely set, flattened spines, which diminished in size and number in the posterior half of the body. The oral sucker was 0.060 to 0.065 mm in diameter; the mouth ventroterminal. There was a distinct prepharynx, about as long as the diameter of the oral sucker. The pharynx was 0.03 to 0.035 mm in diameter. The esophagus was two to three times as long as the pharynx but the length was reduced when the anterior part of the worm was retracted. The caeca met anteriorly at an angle and extended to the posterior end of the body. The genital pore was immediately anterior and somewhat lateral to the acetabulum and opened from a common genital atrium. The testis was single, oval, 0.05 to 0.07 mm in length, median, and situated a short distance posterior to the acetabulum. The cirrus sac was clavate; it contained a large, oval seminal vesicle dorsal to the ovary, a prostate region dorsal to the acetabulum, and a spined ejaculatory duct that may be everted as a cirrus at the genital atrium. The ovary was spherical to oval, 0.025–0.030 mm in diameter, situated just posterior to the acetabulum, and sometimes below the anterior end of the testis. The oviduct arose at the posterior end; it received a short duct from the seminal receptacle and another from the vitelline receptacle, before it entered Mehlis' gland. From the seminal receptacle, Laurer's canal led to the dorsal surface of the body. The vitelline follicles were situated dorsally and laterally at the level of the testis; ducts passed mediad and ventrad to join at the vitelline receptacle. The initial part of the uterus contained spermatozoa and the early coils were filled with transparent spherical to oval eggs with thin membranous walls and intensely stained embryos. Later, the uterus was filled with opaque, operculate, hard-shelled yellow eggs averaging 0.021 by 0.013 mm. The metraterm was short, spined, and opened into the ventral posterior part of the genital atrium. The excretory system was unchanged from the cercarial condition.

Discussion

The species *Lasiotocus elongatus* was included originally in the genus *Genolopa* Linton, 1910, but the validity of the genus is dubious and the species has been transferred to other genera. The taxonomic problem was considered by Stunkard (1981) and need not be repeated. When the asexual generations of species in related genera are available for comparison, the difficulties may be resolved. Cable (1956) described *Cercaria caribbea* XXXVI, a monorchid larva from *Gemma purpurea* taken in Sucia Bay, Cabo Rojo, Puerto Rico. It is very similar to the cercaria of *L. elongata* and undoubtedly the two are closely related, although generic allocation is not feasible.

The monorchid trematodes are parasitic in both marine and freshwater fishes. Their life cycles have been investigated for more than a century (most of the work was done on freshwater species). Many reports noted sexual maturity of metacercarial stages. The studies of Dollfus (1929), Alice Büttner (1951), Chabaud and Biguet (1954) and Biguet *et al.* (1956) have recorded abbreviated and unusual life cycles in many different species. Stunkard (1959) reported that in *Asymphylodora amnicolae* the worms may become gravid in the molluscan host and the vertebrate can be omitted entirely. Although information on life cycles of marine species is meager, there are no reports of progenesis in marine hosts. Is it conceivable that migration of the parasites from marine to freshwater hosts and environments is correlated with early sexual maturity of larval stages?

LITERATURE CITED

ABBOTT, R. T. 1974. *American Seashells*, 2nd Ed. Van Nostrand Reinhold Co., New York. 663 pp.

BIGUET, J., S. DEBLOCK, AND A. CAPRON. 1956. Description d' une métacercaire progénétique du genre *Asymphylodora* Looss, 1899 découvert chez *Bythinia leachi* Sheppard dans le nord de la France. *Ann. Parasitol.*, **31**: 525–542.

BÜTTNER, ALICE. 1951. La progenése chez les trématodes digénétique. *Ann. Parasitol.*, **26**: 19–66, 138–179, 279–322.

CABLE, R. M. 1956. Marine cercariae of Puerto Rico. Sci. Surv. Puerto Rico and Virgin Islands. *New York Acad. Sci.* **16**: 491–577.

CHABAUD, A. G., AND J. BIGUET. 1954. Étude d' un trématode hémiuroide á métacercaire progénétique. I. Développement chez le mollusque. II. Infestation de copépode. III. Développement chez le copépode. *Ann. Parasitol.* **29**: 527–545.

DOLLFUS, R. PH. 1929. Existe-t-il des cycles évolutifs abrégés chez les trématodes digénétique? La cas de *Ratzia parva* (Stossich, 1904). *Ann. Parasitol.* **7**: 196–203.

LINTON, E. 1901. Fish parasites collected at Woods Hole in 1898. *Bull. U. S. Fish Comm. (1899).* **19**: 267–304.

LINTON, E. 1905. Parasites of fishes of Beaufort, North Carolina. *Bull. U. S. Bur. Fish. (1904)* **24**: 321–428.

MANTER, H. W. 1931. Some digenetic trematodes of marine fishes of Beaufort, North Carolina. *Parasitology* **23**: 396–411.

SOBOLEV, A. A. 1955. The family Monorchiidae Odhner, 1911. Pp. 257–464 in K. I. Skrjabin, *Trematodes of Animals and Man.* Vol. **11** Moskva.

STUNKARD, H. W. 1959. The morphology and life-history of the digenetic trematode, *Asymphylodora amnicolae* n. sp.; the possible significance of progenesis for the phylogeny of the Digenea. *Biol. Bull.* **117**: 562–581.

STUNKARD, H. W. 1981. The life history, developmental stages, and taxonomic relations of the digenetic trematode *Lasiotocus minutus* (Manter, 1931) Thomas, 1959. *Biol Bull.* **160**: 146–154.

STUNKARD, H. W., AND J. UZMANN. 1959. The life-cycle of the digenetic trematode, *Proctoeces maculatus* (Looss, 1901) Odhner, 1911 [(syn. *P. subtenuis* Linton, 1906) Hanson, 1950], and description of *Cercaria adranocera* n. sp. *Biol. Bull.* **116**: 184–193.

THOMAS, J. D. 1959. Trematodes of Ghanian sub-littoral fishes. I. The family Monorchiidae. *J. Parasitol.* **45**: 85–112.

Reference: *Biol. Bull.*, **160:** 161–174. (February, 1981)

PROTANDRIC HERMAPHRODITISM IN A MOLE CRAB, *EMERITA ASIATICA* (DECAPODA:ANOMURA)

T. SUBRAMONIAM

Department of Zoology, University of Madras, Madras-600 005, India

ABSTRACT

Protandric hermaphroditism in a mole crab, *Emerita asiatica*, is described. Neotenous males occur at 3.5 mm carapace length (CL) and above, whereas females acquire sexual maturity at 19 mm CL. The neotenous males, as they continue to grow, gradually lose male functions and reverse sex around 19 mm CL. The disappearance of genital papillae is the first visible sign of sex reversal; spermatogonial activity in testes ceases but hyperactivity of the mesodermic cells ensues. In the CL range of 19–22 mm, the male's gonad comprises inactive testicular and active ovarian portions; the median ovarian limb beyond the fused posterior extremity of the testes lacks testicular elements; and the vas deferens is intact but a pair of functional oviducts is formed. These intersexuals possess three pairs of pleopods. A few have eggs, and thus constitute secondary females in the population. Androgenic glands, active in the neotenous males, show signs of degeneration in the larger males, and disappear in the intersexuals. The mesodermic cells of the gonad undergo important functional changes during sex reversal. Sex-changers with incomplete transformation of testis into ovary and imperfectly differentiated oviducts are also reported.

INTRODUCTION

In mammals the histogenesis of the gonad as an ovary or testis is determined by the sex genes that initiate chemical synthesis of substances responsible for sexual differentiation (Witschi, 1971). The malacostracan crustaceans are generally gonochoristic with genetically determined sex. But several of them exhibit both functional and non-functional hermaphroditism. Functional protandric hermaphroditism has been well documented in the deep-sea prawns and two isopod groups, *Cymathoides* and *Cryptoniscidae* (Yaldwyn, 1966; Charniaux-Cotton, 1975a). Here, each sex possesses complete genetic information for the morphogenesis of both sexes. The genes for male morphogenesis act in the presence of an androgenic gland hormone and for females in its absence (Charniaux-Cotton, 1960a).

Other groups of crustaceans may well show change of sex with growth, but evidence is rather meager. Mole crabs, including various species of *Emerita*, are typical burrowing forms on the wave-washed sandy beaches of temperate and tropical seas. Several species show sexual dimorphism in size. For example, the males of *E. asiatica* acquire sexual maturity soon after their metamorphosis from megalopa larvae (3.5 mm carapace length, CL) while the females attain it after considerable growth (19–22 mm CL) (Subramoniam, 1977). This size distinction at sexual maturity of *Emerita* is considered a characteristic feature of the genus by Efford (1967). Barnes and Wenner (1968), on the contrary, proposed a protandric hermaphroditism based on sex-ratio studies on *E. analoga*. However, the sex ratio

Received 14 January 1980, accepted 3 October 1980.
Abbreviations: CL, carapace length.

studies of Wenner (1972) failed to strengthen the likelihood of a sex reversal in *E. asiatica*, the overlap in size range between males and females being too wide to suggest sex reversal (Subramoniam, 1977). The neotenic males grow up to 11 mm CL without reduction in size of the gonadal apparatus. Judging from the linear increase of the male gonadal apparatus with general body growth, Subramoniam believed that the neotenic males of *E. asiatica* do not change sex as they grow.

The present paper dealing with *E. asiatica* reports new data on the continued growth of larger males, which pass through an intersexual phase before some obtain complete functional protandric hermaphroditism.

Materials and Methods

Specimens of *Emerita asiatica* Milne Edwards, belonging to the family Hippidae (Decapoda:Anomura), were collected from the intertidal regions of the Marina beach, opposite the Madras University buildings, Madras, India. This crab has distinctly zoned habitats in the intertidal region, the smallest individuals being commonest in the fine sand near the high water mark and the largest in coarse sand near the low water mark. Between these two zones, specimens of intermediate sizes are found (Alikunhi, 1944; Subramoniam, 1979a). At least 60 crabs 3.5–33 mm CL were hand-picked every fortnight and brought to the laboratory.

Male and female *Emerita* specimens were distinguished following the descriptions given by Subramoniam (1977). Genital papillae at the base of the coxae of the fifth thoracic leg identified males. The females were recognized by the occurrence of three pairs of ovigerous pleopods in the abdomen. Specimens of both the sexes were dissected to observe the developmental stages of reproductive organs. For histological studies, the gonad was fixed in Bouin's and neutral formaldehyde. Paraffin sections were cut 6 μm thick and stained in haematoxylin and eosin.

Results

Reproductive structures of primary females and males

In females, the ovary is a paired organ lying partly in the thorax and partly in the abdomen. The anterior ends of the ripe ovary consist of two lobes each, one extending forward and the other to the side. The posterior parts of the paired ovary are connected by a transverse bridge of ovarian tissue lying over the pyloric region of the stomach. Behind the posterior paired parts, the ovary is fused to form a median cord which extends to the fifth abdominal segment (Fig. 1a). A pair of oviducts take their origin from the middle region of the paired posterior limb and open at the base of the coxae of the third thoracic leg. Externally, there are three pairs of pleopods in the ventral region of the first three abdominal segments.

In males too, the testis is paired but fused at the posterior extremity, a region corresponding to the posterior fusion of the ovarian limbs. The prominent vasa deferentia originate from the region where the limbs of the testes fuse (Fig. 1b) and open into the genital papillae at the base of the coxae of the fifth thoracic leg. There are no pleopods in the abdominal segments.

In neotenous males the testis is composed of several acini. The spermatogonial cells border the wall of the acinus while the spermatocytes and developing spermatozoa occupy the lumen. But these are so crowded in the active stage that the regular acinar arrangement is not often clearly demarcated (Fig. 2). Non-germinal somatic mesodermal cells are rare, and are found only between the closely packed

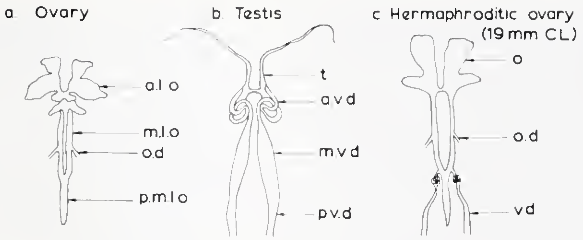


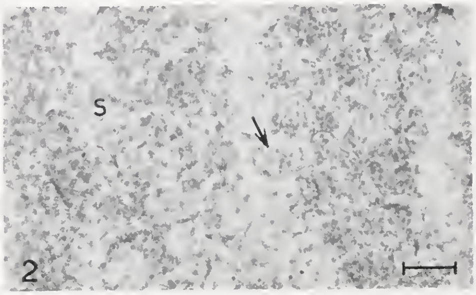
FIGURE 1. Diagrammatic representation of the testis, ovary, and the hermaphroditic ovary. t = testis; a.v.d. = anterior vas deferens; m.v.d. = mid vas deferens; p.v.d. = posterior vas deferens; a.l.o. = anterior ovarian lobe; m.l.o. = middle ovarian lobe; o.d. = oviduct; p.m.l.o. = posterior median ovarian limb; o = ovary; v.d. = vas deferens.

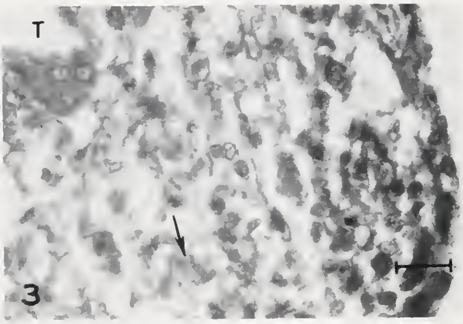
acini. In primary females—crabs that are female at the outset—the pleopods begin to appear at an early stage, ±12 mm CL (Subramoniam, 1977). Similarly, a pair of female gonopores appears at ±10 mm CL in primary females, whereas in the secondary females, originally males, they appear at ±19 mm CL.

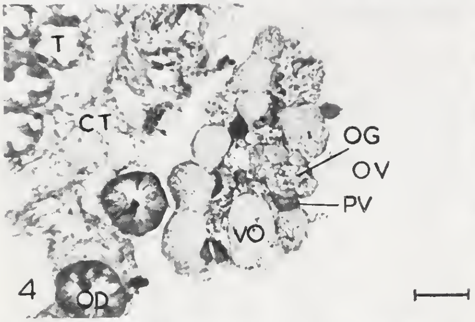
Gonadal changes during sex reversal

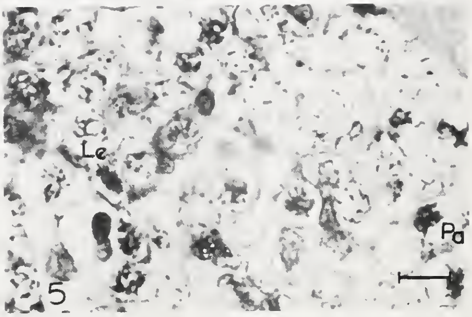
Contrary to earlier observations (Subramoniam, 1977), males above 11 mm CL have been recorded. In them, the genital papillae gradually become smaller. The testes and vas deferens are intact and do not regress in size. However, histological sections of the testis of a 15 mm CL male whose genital papilla is completely reduced show remarkable changes in the cellular organization (Fig. 3). Testicular acini lined with spermatogonial cells are confined to a few places, and they do not contain spermatocytes and spermatids. As the testis loses its function, the somatic mesodermal cells become hyperactive and multiply to occupy the whole space made available by the shrinking testicular acini. These cells increase in size. They contain a large quantity of acid muco-polysaccharides as determined by histochemical tests. The vas deferens contains the spermatozoa and the spermatophoric ribbon. There is no evidence for any oocyte differentiation in the testis.

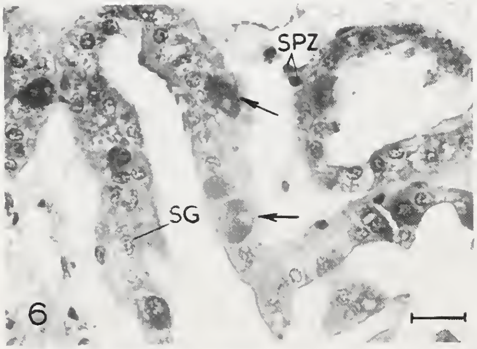
Though testicular activity ceases completely around 15 mm CL, sex reversal appears to occur only in the size range 19–22 mm CL. Although sex-reversed crabs resemble females externally (genital papillae absent; pleopods well formed and sometimes with eggs), they possess a hermaphroditic ovary with testicular portions intact. Curiously enough, in one such crab, measuring 19 mm CL, the paired testis portion was white, but along its mid-dorsal line were strings of golden-yellow oocytes. The posterior median cord is always yellow. A cross section through the testicular limb shows that it comprises two well-defined portions: the testicular acini and an ovarian region enclosing a central cluster of oogonial cells surrounded by oocytes in generative, post-pachytene, previtellogenic, and yolk-laden vitellogenic phases (Fig. 4). The germinal zone of the ovary contains very actively dividing oogonial cells, and the primary oocytes surrounding the germinal zone show features of early prophase of the first meiotic division (Fig. 5). By contrast, the testicular acini have no spermatocytes in the lumen, nor do the spermatogonial cells bordering the acini show any sign of activity. The nuclei of these resting spermatogonial cells contain clumps of chromatin. However, some of these cells are enlarged, with basophilic cytoplasm characteristic of primary oocytes in the premeiotic stage (Fig. 6). Mesodermic cells are not found in the testicular portion, but are seen in large

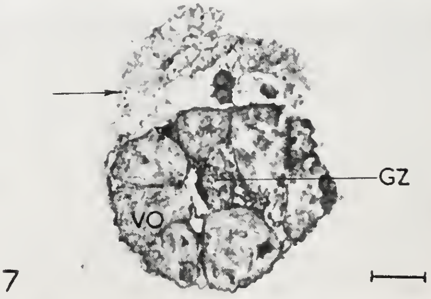


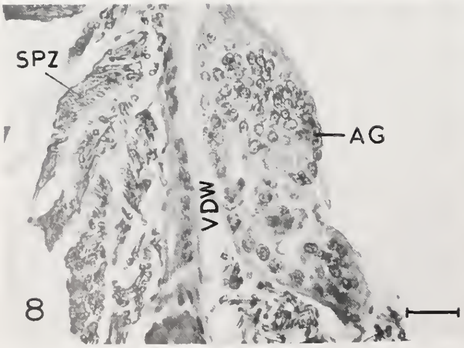












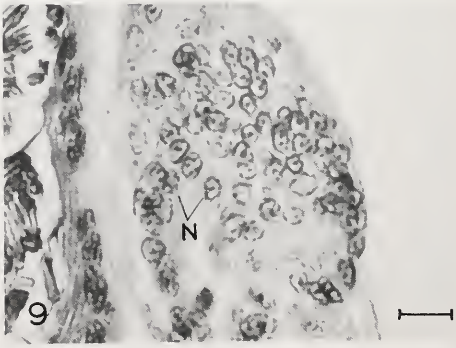


FIGURE 2. Section through the testis of an active male (5 mm CL) showing the closely packed acini (arrow) with developing spermatozoa (s) in them. Bar = 20 μm.

FIGURE 3. Transverse section through testis of a large male (CL 15 mm) indicating hypertrophy of the somatic mesodermal cells (arrows). T = testicular acinis. Bar = 20 μm.

FIGURE 4. Section of a hermaphroditic ovary of *Emerita asiatica* (19 mm CL). The testicular acini (T) contain spermatogonial cells, the lumen is devoid of spermatocytes and spermatozoa. The

numbers in the ovarian region; the vitellogenic oocytes are, in fact, surrounded by them. Pockets of primary gonial cells have also been observed in the testicular region.

The median posterior limb of the ovary is devoid of testicular elements but possesses a basal germinal zone with previtellogenic and vitellogenic oocytes arranged centripetally. The median ovarian cord at the point of its origin contains the basal laminar tissues without any testicular portion attached (Fig. 7) although the more posterior part of this ovarian limb contains only ovarian cells. In several such hermaphroditic females the vas deferens was intact and contained spermatophores, although the genital papillae through which the vas deferens opens were completely sealed off. A pair of functional oviducts formed anterior to the vas deferens had openings on the base of the third walking legs. Sixteen specimens of such intersex crabs were obtained in October–November 1978 in the size range of 19–22 mm CL. Similar forms were also obtained in subsequent collections. The occurrence of such secondary females was recorded earlier in a population of *Pandalus borealis* (Carlisle, 1959).

Androgenic gland and changes in functional morphology during sex reversal

The androgenic gland of *Emerita* has not been previously described. In reproductively active males (±5 mm CL) it is simple cellular strands, as in brachyuran crabs such as *Carcinus maenas* (Charniaux-Cotton *et al.*, 1966). These strands lie in the subterminal portion of the distal vas deferens. The gland is composed of compactly packed columnar cells (Fig. 8), whose nuclei are ovoid, conspicuous, and endowed with dense chromatin (Fig. 9). Dense secretory materials with vacuoles of varying sizes fill the cytoplasm. The lobes of the androgenic gland are either spread out on the distal regions of the vas deferens or hang freely in the haemocoel with their bases attached to the outer layer of the vas deferens.

By contrast, the androgenic glands of a larger male (CL 9 mm) show many degenerative changes (Fig. 10). The majority of the glands have degenerated and a fine granular central portion without evidence of the normal cellular structure is evident. On one side are many pycnotic nuclei without any trace of cell walls, suggesting recent disintegration of cells (Fig. 11). However, new cordons of cells proliferate on the periphery of the gland, mostly in the basal region with which it is attached to the vas deferens wall. The cells of the new cordons are smaller than those of the androgenic gland of neotenic males. The degenerative areas of the

---

ovarian part (OV) is separated by strands of connective tissue (CT). The oogonial cells (OG) are in the center surrounded by previtellogenic (PV) and vitellogenic (VO) oocytes. OD = Oviduct. Bar = 100 μm.

FIGURE 5. Higher magnification of germinal zone. Nuclei of many oocytes in the generative phase show thread-like chromosomes believed to represent leptotene (Le) and pachytene (Pa) stages. Bar = 10 μm.

FIGURE 6. Testicular acini showing oocytes in previtellogenesis (arrow). Note the basophilic cytoplasm of the previtellogenic oocytes. Spermatogonial cells (SG) are in the resting stage. Follicular cells are absent and a few residual spermatozoa (SPZ) are present. Bar = 25 μm.

FIGURE 7. Cross section through the proximal region of posterior median cord of the ovary. Germinal zone in the centre (GZ) surrounded by vitellogenic oocytes (VO). The connective tissue basal lamina contain many follicle cells (arrow). Bar = 100 μm.

FIGURE 8. Transverse section through the androgenic gland of a neotenous male (5 mm CL) AG = androgenic gland; VDW = vas deferens wall; SPZ = spermatozoa. Bar = 30 μm.

FIGURE 9. Higher magnification of the androgenic gland. Note the hyperactive nuclei (N) with dense basophilic material clumping on the nuclear wall. Bar = 13 μm.

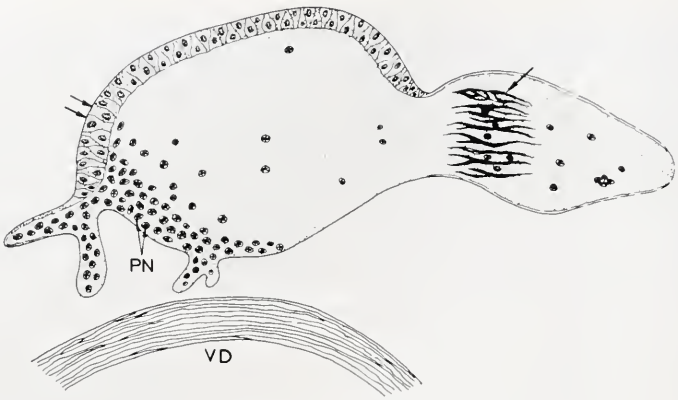


FIGURE 10. Composite diagram of androgenic gland of a large non-functional male (CL 9 mm). Large portion of the gland shows cellular degeneration (stippling). Scar-like thickenings (single arrow) are evident in the distal region of the gland. New cordon of cells appears in the basal region (double arrow). Figure not drawn to scale. V.D. = vas deferens; P.N. = pycnotic nuclei.

gland conform to the descriptions by Charniaux-Cotton (1960b, 1962) and Payen (1972). At the distal end of the gland are scar-like thickenings (Fig. 10). This region is devoid of regenerating cordons of cells; pycnotic nuclei have wheel-shaped chromatin clumps adhering to the nuclear membranes. At 15 mm CL and above, the androgenic gland was not detected.

Observation on incomplete sex reversal

Sexual reversal is not always complete; in some forms the males fail to become functional secondary females and remain intersexual. Two large crabs (CL 28, 29 mm) with well developed female secondary sexual characters (three pairs of pleopods and a pair of gonopores on the coxae of the third leg) had hermaphroditic ovaries, which were white and very thick. Interestingly, the vas deferens is retained in such crabs although the genital papillae are absent. Histological examination of the ovary revealed that the sex reversal in these crabs was incomplete in that the separate ovarian portion never formed. The anterior half of the paired tubular gonad was dominated by oocytic differentiation whereas the posterior half possessed inactive testicular tissues. Sections of the anterior region of the gonad show vitellogenic oocytes arranged on the dorsal periphery of the testis (Fig. 12). Deeper, below the vitellogenic oocytes, lie the previtellogenic oocytes. The follicle cells surrounding the previtellogenic oocytes are very active. However, the vitellogenic oocytes are invaded by enlarged follicle cells (Fig. 12) involved in resorption of yolk products. That this process of yolk absorption is by way of enzymatic conversion is shown by the staining reactions of the yolk globules and the contents of the follicle cells inside the ooplasm. The yolk contents are eosinophilic whereas the corresponding granules in the follicle cells are basophilic. This suggests that the yolk products are dissolved by some hydrolytic enzyme released from the follicle cells dissolves the yolk products, engulfing them intact and storing them. Delamination of follicle cells into yolk-laden oocytes can also be seen occasionally (Fig. 13). The testicular region contains several acini, each lined by inactive spermatogonial cells. The lumen is also empty of differentiated gametes. The follicle cells in the anterior ovarian region are of normal size and shape, whereas those in the testicular region are hyperactive (Fig. 14). A transverse section through the oviduct (Fig. 15) originating from the ovarian region shows that its differentiation is incomplete in that it does not acquire a lumen and remains as strands of tissues. This condition is in contrast to the well differentiated functional oviduct of the secondary female (Fig. 4). The gonadal composition described so far gradually changes towards the posterior region. Here, no vitellogenic oocytes are found and the number of previtellogenic oocytes is also limited. Much of the gonadal portion is occupied by the testicular acini, which in several regions are filled with mucous secretions (Fig. 16). Such mucus secretions are also found on the periphery of the gonad (Fig. 17). The proximal vas deferens originating from the posterior region of the gonad is intact and its lumen contains residual sperm cells mixed with luminal secretions (Fig. 17). Neither spermatocytes nor spermatozoa, however, are found in the gonad.

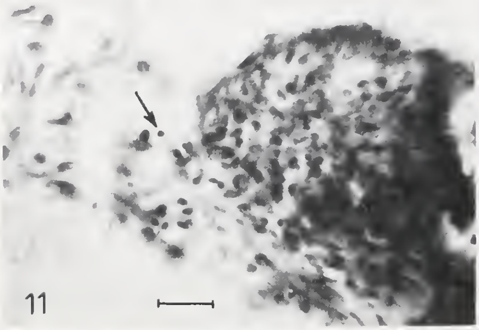
Discussion

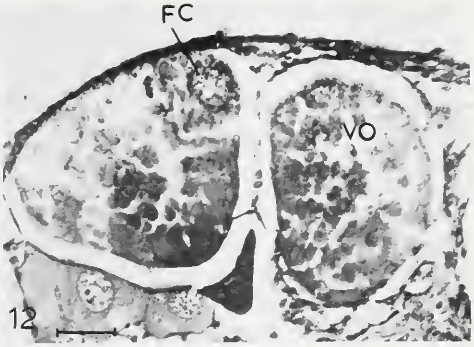
It is evident from the table listing the size ranges of mature male and female of different species of *Emerita* that precocious sexual maturity of males, leading to neoteny, is widespread. However, the neotenous males tend to grow to larger sizes (Table 1). Size-related sex ratio curves in a well studied species, *E. analoga*, have led to the proposition of sex reversal (Barnes and Wenner, 1968; Wenner, 1972). Similar sex-ratio studies on *E. asiatica* males and females of different sizes, including an analysis of post-larval juveniles, do not support the contention of sex reversal (Subramoniam, 1977). Subramoniam suggested that the prevalence of males in the smaller size classes only indicated different growth rates between males and females, and that the absence of males in the larger size classes was due to mortality. More recently, Haley (1979) came to a similar conclusion from evidence he obtained that males of smaller size classes molt less frequently than females of the corresponding size in a hippid mole crab, *Hippa pacifica*.

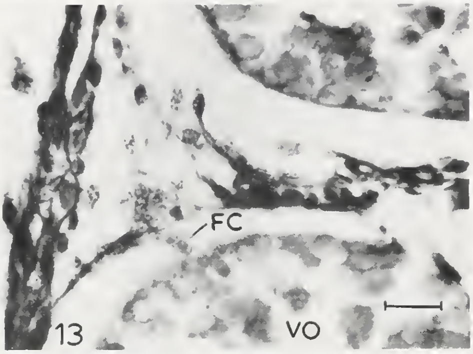
The histological results reported in the present study demonstrate unequivocally the existence of functional protandric hermaphroditism in *Emerita asiatica*. Sex reversal in this crab is briefly reported elsewhere (Subramoniam, 1979b). The main reproductive events enumerated in Figure 18 indicate that neotenous males continue to grow after serving a normal male life (Subramoniam, 1977), deviate from normal sexual behaviour (Subramoniam, 1979c), gradually lose their secondary and primary sexual characters, and undergo sexual reversal by acquiring female characters at ± 19 mm CL. As a result of this, the population consists of two kinds of females: primary and secondary. Interestingly, the sex reversal of males into females occurs at the same size-range as sexual maturity of the primary females, which do not pass through a male phase (Fig. 18). Such a pattern of sexuality in the life history of *E. asiatica* has great adaptive significance by vastly augmenting the capacity to reproduce and to colonize the turbulent sandy intertidal zone. The precocious maturation of males that results in neoteny facilitates mating in the intertidal region without disturbing the normal activity of the females. Sex reversal, as exemplified by protandric hermaphroditism, increases the number of egg-laying females by adding secondary females to the female population.

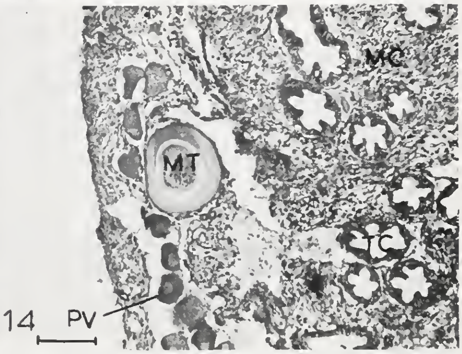
Although environmental conditions may encourage the reproductive strategy, it is physical ease of sex reversal that lets it occur so frequently in malacostracean crustaceans.

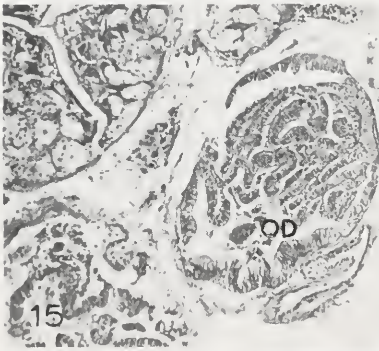
The location of the germinal zone in the hermaphroditic crustaceans has long been debated. It is described either as the cortical germinal epithelial layer (*P. platyceros*, Hoffman, 1972) or as a single germinal zone at the basal lamella (*P. borealis* and *Lysmata seticaudata*; Charniaux-Cotton, 1975a). The hermaphroditic

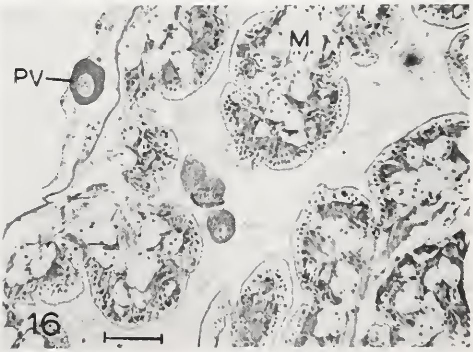

11


FC
VO
12


FC
13
VO


MC
MT
TC
14
PV


OD
15


M
PV
16

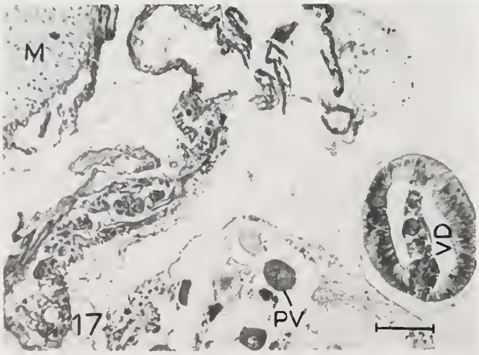

M
VD
17
PV

TABLE 1

Stage of sexual maturity of male and female Emerita *species.*

Species	Reference	Carapace length range of mature *Emerita* in mm: Male	Female
E. analoga	Knox and Boolootian, 1963	10.0–22.0	15.0–30.0
E. analoga	Efford, 1967	6.0–12.0	13.0–31.0
E. analoga	Barnes and Wenner, 1968	6.0–11.0	8.0–22.0
E. asiatica	Menon, 1933	3.5–7.5	22.0–30.0
E. asiatica	Subramoniam, 1977 and present study	3.5–15.0	19.0–33.0
E. austroafricana	Barnard, 1950	*–35.0	23.0–37.0
E. holthuisi	Sankolli, 1965	11.0–17.0	12.0–18.0
E. holthuisi	Achuthankutty and Wafar, 1976	*–10.0	10.0–15.3
E. portoricensis	Goodbody, 1965	*–8.0	9.0–17.0
E. rathbunae	Efford, 1967	2.5–**	33.0–41.0
E. talpoida	Wharton, 1942	3.8–14.0	*–26.0
E. talpoida	Efford, 1967	2.5–12.0	14.0–29.0

* Minimum size not given.
** Maximum size not given.

ovary of *E. asiatica* reveals yet another mode of gonial-cell proliferation in both male and female phases. In the active testis, spermatogonial cells border the testicular acini, into which the developing male gametes are packed closely. During sex reversal the oogonial cells are in an ovarian anlagen outside the testicular region and separated from it by a thick basement membrane. It is not clear whether the spermatogonial cells from the acini migrate through the epithelial barrier, turning into oogonial cells, or whether undifferentiated gonial cells already in the testis give rise to oogonial cells. That the spermatogonial cells in the testicular acini are capable of giving rise to oogonial cells is evidenced by the precocious differentiation of several spermatogonial cells into oocytes in the testicular acini. This recalls a condition found in the non-functional hermaphroditic crayfish, *Pontastacus leptodactylus leptodactylus*, where the spermatogonial cells in testicular acini give rise to oocytes when the androgenic glands are very small (Payen, 1973). In in-

---

FIGURE 11. Magnification of area shown in Figure 10 in the regions of cellular disintegration. Note the concentration of pycnotic nuclei (arrows) without cell boundary. Bar = 25 μm.

FIGURE 12. Transverse section through the anterior region of the hermaphroditie ovary of an intersexual (CL 28 mm) showing the row of vitellogenic oocytes (VO) on the dorsolateral region. Note the invasion of follicle cells (FC) digesting the yolk. Bar = 25 μm.

FIGURE 13. Showing the delamination of the follicle cells (FC) into the vitellogenic oocytes (VO). Bar = 10 μm.

FIGURE 14. Transverse section through the middle region of a hermaphroditic ovary of an intersexual (CL = 28 mm) to show the hyperactive somatic mesodermal cells (MC). TC = Testicular acini; MT = metacercaria; PV = Previtellogenic oocytes. Bar = 150 μm.

FIGURE 15. Transverse section through the oviduct of an intersexual (CL 29 mm) to show that it is in an undifferentiated condition. OD = Oviduct. Bar = 150 μm.

FIGURE 16. Transverse section through the hermaphroditic ovary of an intersexual (CL 29 mm) in the middle region. The testicular acini is filled with mucus (M). A few previtellogenic oocytes (PV) are also present. Bar = 100 μm.

FIGURE 17. The posterior-most region of the gonad of a large intersexual (CL 28 mm) indicates the presence of thick mucous coating (M). It also shows the vas deferens (VD) with sperm mass substance inside it. PV = Previtellogenic oocytes. Bar = 25 μm.

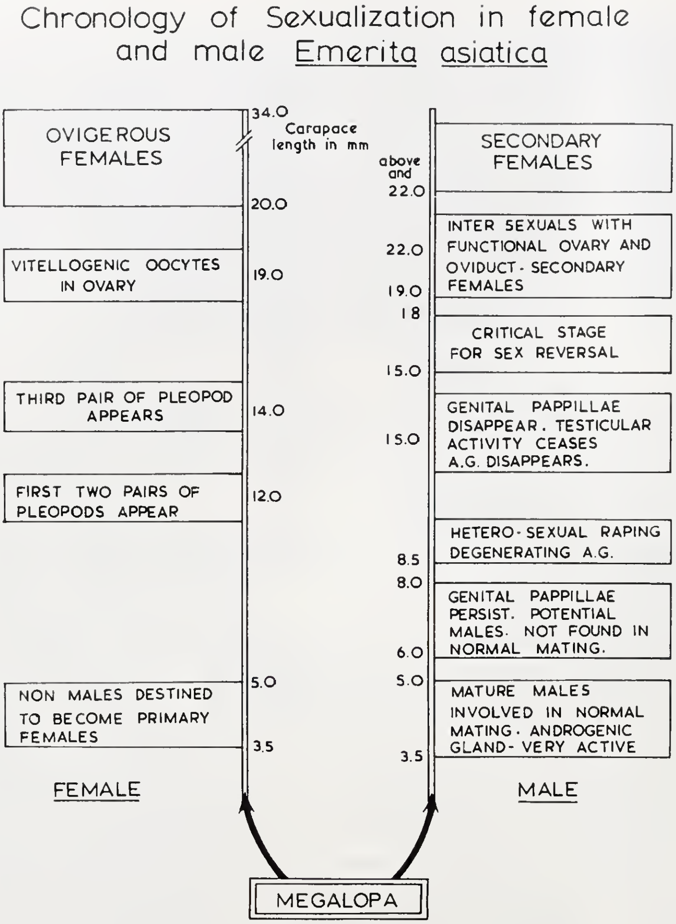

Chronology of Sexualization in female and male Emerita asiatica
OVIGEROUS FEMALES
34.0
Carapace length in mm
20.0
VITELLOGENIC OOCYTES IN OVARY
19.0
THIRD PAIR OF PLEOPOD APPEARS
14.0
FIRST TWO PAIRS OF PLEOPODS APPEAR
12.0
NON MALES DESTINED TO BECOME PRIMARY FEMALES
5.0
3.5
FEMALE
above and 22.0
SECONDARY FEMALES
22.0
19.0
INTER SEXUALS WITH FUNCTIONAL OVARY AND OVIDUCT - SECONDARY FEMALES
18
15.0
CRITICAL STAGE FOR SEX REVERSAL
15.0
GENITAL PAPPILLAE DISAPPEAR. TESTICULAR ACTIVITY CEASES A.G. DISAPPEARS.
8.5
HETERO-SEXUAL RAPING DEGENERATING A.G.
8.0
6.0
GENITAL PAPPILLAE PERSIST. POTENTIAL MALES. NOT FOUND IN NORMAL MATING.
5.0
3.5
MATURE MALES INVOLVED IN NORMAL MATING. ANDROGENIC GLAND - VERY ACTIVE
MALE
MEGALOPA

FIGURE 18.

complete sex reversals in *E. asiatica*, a separate ovary is not formed but oocytes differentiate in the dorsolateral periphery of the inactive testis. Alternatively, therefore, it is possible that male and female gonocytes germinate in a common zone in the dorsal surface of the testis all along its length, giving rise to testicular acini early in development. In *P. borealis* and *L. seticaudata*, oogenesis does proceed to previtellogenesis during the male phase, while the active spermatogonia move out from both sides of the ventrally placed germinal zone to surround the ovarian core (Charniaux-Cotton, 1975a).

In *P. borealis*, Allen (1959) has also shown that all prawns showing external male characters have an ovarian germinal strand. Such an intersex condition in gonadial activity has not been observed in the present study on *E. asiatica*. In fact, the ovarian transformation of the testis does not occur soon after the stoppage of spermatogonial activity. Instead, there is a period of quiescence of the gonocytes. Again, a pair of thin walled oviducts with distinct gonopores are present during the male phase in *P. platyceros*. But in *E. asiatica* the secondary female sexual characters do not appear until after the formation of the ovarian anlagen. The oviducts are differentiated only in intersexuals 19 mm CL and larger. Interestingly, in incomplete intersexuals of 28–29 mm CL the oviduct remains incompletely differentiated, resulting in failure of ovulation.

An important feature of sex reversal in *E. asiatica* is the change in the functional morphology of the somatic mesodermal cells of the gonad. During the active male phase these somatic mesodermal cells are small, and confine themselves to the interspaces between the testicular acini. Once the testis loses its function, the mesodermal cells become hyperactive. Such hyperactivity of the mesodermal cells in the testis during periods of sexual inactivity has also been shown in *P. leptodactylus leptodactylus* (Amato and Payen, 1978). During sex reversal, following the formation of separate ovarian anlagen, the ovarian structure contains typical follicle cells that have either migrated from the testicular region or differentiated from a pre-follicular mesodermic tissue of the gonad. These cells characteristically attach themselves to the vitellogenic oocytes and are flattened on the oocyte surface, probably mediating uptake of vitellogenic proteins from the hemolymph (Charniaux-Cotton, 1975b). In the partly transformed hermaphroditic females of *E. asiatica*, however, the mesodermic cells are numerous, and they produce a thick mucous coat around the posterior testicular portion of the gonad. In addition, the lumina of the testicular acini are filled with such secretions. These substances are probably secreted by the mesodermic cells of the testes. It is of interest to note here that the ovarian follicle cells of a hermit crab, *Clibanarius clibanarius*, are also rich in acid mucopolysaccharide substances (Varadarajan and Subramoniam, 1980).

In incomplete transformation of the gonad, the follicle cells around the vitellogenic oocytes occupying the anterior region of the gonad assume the function of enzymatic digestion of the yolk materials by invading the ooplasm. Follicle cells participating in the oosorptive process have been observed in several crustacean species (Carayon, 1941; Baffoni, 1962).

All this implies an important role for mesodermal cells in the sex reversal of *E. asiatica.* It is tempting to suggest a strong influence of androgenic gland hormone on the activity of these cells as indicated by its apparent disappearance in males above 15 mm CL, possibly ushering in sex reversal.

It is generally accepted that inversion of the sexual phenotype is influenced by epigamous factors exerted during growth (Gallien, 1959). In malacostracan crustaceans the hermaphroditic potentialities are governed by the androgenic gland hormone (Charniaux-Cotton, 1965a, for references). The sequential disappearance of primary and secondary male characters during the changeover phase and the concomitant assumption of female characters strongly suggest similarities with protandric hermaphroditic natantians such as *P. borealis* and *L. seticaudata* with regard to androgenic gland control of sexual differentiation. A high androgenic gland activity when the neotenous males are reproductively active, and the degeneration of the androgenic gland during the gradual disappearance of male secondary sexual characters and stoppage of spermatogonial activity in larger males, clearly suggest that a fall in circulating androgenic gland hormone may be responsible for these changes in *E. asiatica.* Extirpation and grafting experiments of androgenic gland in *L. seticaudata* have yielded similar results (Charniaux-Cotton, 1959, and Berrear-Bonnenfant, 1963).

Touir (1973) brought forward new data that in hermaphroditic natantians the circulating androgenic gland hormone determines the appearance, growth, and maintenance of male external sex characters, whereas in gonochoristic decapods the gland is only capable of provoking the appearance of these characters, and is not needed to maintain them. More recently, Touir (1977a, b, c) has provided experimental evidence that in the hermaphroditic prawn *L. seticaudata*, this hormone is not indispensable for spermatogenesis, but that it regulates intensity of spermatogenic activity as a result of its mitogenic action on the gonia. A factor from the brain is said to maintain the male genital apparatus. Another factor from the protocerebrum is suggested to be responsible for the maintenance of the androgenic gland, and the absence of this factor causes the disappearance of the gland in *L. seticaudata*. In the light of these observations it may be suggested that the sex reversal in *E. asiatica* is brought about by the disappearance of the above two protocerebral neurosecretory factors.

The incomplete transformation of sex in some large crabs may corroborate this bihormonal influence of sex reversal. In these animals, the neurosecretory substance controlling the spermatogonial activity may disappear completely, so that spermatogonial activity stops. At the same time, the autodifferentiation of oocytes in the anterior half of the paired testis may support the existence of gonadal territoriality in responding to the androgenic gland hormone, as proposed by Legrand (1964). The implication is that a low titer of the hormone prevents the oocytes from reaching previtellogenesis, by inhibiting the folliculogenesis in the posterior testicular territory. On the other hand, the anterior ovarian region, free from the gland's influence, may allow oocytes to undergo vitellogenesis but because of the incomplete oviductal differentiation, oosorption occurs with the help of follicle cells. The retention of the vas deferens in these crabs suggests that the gland has not completely disappeared, thus preventing the complete transformation of sex. Experimental evidence along these lines is highly desirable.

Protandric hermaphroditism is not uncommon among Malacostraca. However, this record of the phenomenon in *E. asiatica* is significant and points to the high probability of sex reversal in such species of *Emerita* where the males attain precocious sexual maturity in the post-larval stage.

ACKNOWLEDGMENTS

I thank Prof. K. Ramalingam for provision of facilities and K. Uma, S. Ezhilarasi, and K. Natarajalingam for their help. This work was aided by a grant F. 25.5 (7790) 79 (SR.1) from U.G.C.

LITERATURE CITED

Achuthankutty, C. T., and M. V. M. Wafar. 1976. Ecology of sandy beach at Sancoale, Goa: Part II–Population model and production of *Emerita holthuisi* Sankolli. *Indian J. Mar. Sci.* **5:** 98–102.

Alikunhi, K. H. 1944. Zonal distribution of *Emerita. J. Bombay Nat. Hist. Soc.* **45:** 94–96.

Allen, J. A. 1959. On the biology of *Pandalus borealis* Kroyer with reference to a population of the Northumberland coast. *J. Mar. Biol. Assoc. U. K.* **38:** 189–220.

Amato, G. D., and G. G. Payen. 1978. Mise en évidence du controle endocrine des différentes étapes de la spermatogenise chez l'ecrevisse *Pontastacus leptodactylus leptodactylus* (Eschscholtz, 1823) Crustacé, Decapode, Reptantia. *Gen. Comp. Endocrinol.* **36:** 487–496.

Baffoni, G. M. 1962. Sur le assorbimento degli ovociti in un Crostaceo decapode di acquadolce *Acta Embryol. Morphol. Exp.* **5:** 124–125.

Barnes, N. B., and A. M. Wenner. 1968. Seasonal variation in the sand crab *Emerita analoga* (Decapoda, Hippidae) in the Santa Barbara area of California. *Limnol. Oceanogr.* **13:** 465–476.

Berreur-Bonnenfant, J. 1963. Autodifferentiation ovarienne dans les gonades males de Crustaces eu Culture *in vitro. Bull. Soc. Zool. Fr.* **88:** 235–238.

Barnard, K. H. 1950. Descriptive catalogue for South African decapod Crustacea. *Ann. S. Afr. Mus.* **38:** 1–837.

Carayon, J. 1941. Morphologie et structure de l'appareil genital female chez quelques pagures. *Bull. Soc. Zool. Fr.* **66:** 95–122.

Carlisle, D. B. 1959. On the sexual biology of *Pandalus borealis* (Crustacea Decapoda). The termination of the male phase. *J. Mar. Biol. Assoc. U. K.* **38:** 481–491.

Charniaux-Cotton, H. 1959. Masculinisation des femalles de la Crevette à hermaphrodisme Protérandrique *Lysmata seticaudata*, Par greffe de glandes androgènes. Interprétation de l'hermaphrodisme chez les Décapodes. Note prélimineire. *C.R. Acad. Sci. Paris* **249:** 1580–1582.

Charniaux-Cotton, H. 1960a. Sex determination. Pp. 411–447 in T. H. Waterman, Ed., *The Physiology of Crustacea*, Vol. I. Academic Press, New York.

Charniaux-Cotton, H. 1960b. Physiologie de l'inversion sexuelle chez la Crevette à hermaphrodisme protérandrique functionnel, *Lysmata seticaudata. C.R. Acad. Sci. Paris* **250:** 4046–4048.

Charniaux-Cotton, H. 1962. Androgenic gland of crustaceans. *Gen. Comp. Endocr.* **8:** 241–247.

Charniaux-Cotton, H. 1975a. Hermaphroditism and gynandromorphism in malacostracan Crustacea. Pp. 91–105 in R. Reinboth, Ed., *Intersexuality in the animal kingdom.* Springer-Verlag, Berlin.

Charniaux-Cotton, H. 1975b. L'ovogenèse et sa regulation chez les crustacés supérieurs. *Ann. Biol. Anim. Bioch. Biophys.* **15:** 715–724.

Charniaux-Cotton, H., C. Zerbib, and J. J. Meusy. 1966. Monographie de la glande androgène des Crustacés supérieurs. *Crustaceana* **10:** 113–136.

Efford, I. E. 1967. Neoteny in sand crabs of the genus *Emerita* (Anomura, Hippidae). *Crustaceana* **13:** 81–93.

Gallien, L. 1959. Sex determination. Pp. 399–446 in S. Brachet and Mirsky, A. E., Ed., *The Cell. Biochemistry, Physiology and Morphology.* Vol. I. Academic Press, New York.

Goodbody, I. 1965. Continuous breeding in populations of tropical crustaceans, *Mysidium columbiae* (Zimmer) and *Emerita portoricensis* Schmidt. *Ecology* **46:** 195–197.

Haley, S. R. 1979. Sex ratio as a function of size in *Hippa pacifica* Dana Crustacea, Anomura, Hippidae. A test of the sex reversal and differential growth rate hypothesis. *Am. Nat.* **113:** 391–397.

Hoffman, D. L. 1972. The development of the ovotestis and copulatory organs in a population of protandric shrimp, *Pandalus platyceros* Brandt from Lopez sound, Washington. *Biol. Bull.* **142:** 251–270.

Knox, C., and R. A. Boolootian. 1963. Functional morphology of the external appendages of *Emerita analoga. Bull. S. Cal. Acad. Sci.* **62:** 45–68.

Legrand, J. J. 1964. La différentiation sexualle chez les Oniscoides gonochorique. *C.R. Seances Soc. Biol. Fil.* **158:** 340–343.

Menon, M. K. 1933. The life histories of decapod Crustacea from Madras. *Bull. Madras Govt. Mus. (N.S.)* **3:** 1–45.

Payen, G. 1972. Etude ultrastructurale de la dégénérescence cellulaire dans la glande androgène du Crabe *Ocypode quadrata* (Fabricius) *Z. Zellforsch.* **129:** 370–385.

Payen, G. 1973. Étude descriptive des principales étapes de la morphogenèse sexuelle chez un Crustacé Décapode a développement condensé, l'écrevisse *Pontastacus leptodactylus leptodactylus* (Eschscholtz, 1823) *Ann. Embryol. Morphol.* **6:** 179–206.

Sankolli, N. K. 1965. On a new species of *Emerita* (Decapoda, Anomura) from India with a note on *E. emeritus* (L). *Crustaceana* **8:** 48–54.

Subramoniam, T. 1977. Aspects of sexual biology of the anomuran crab *Emerita asiatica. Mar. Biol.* **43:** 369–377.

Subramoniam, T. 1979a. Some aspects of reproductive ecology of a mole crab, *Emerita asiatica* Milne Edwards, *J. Exp. Mar. Biol. Ecol.* **36:** 259–268.

Subramoniam, T. 1979b. Sex reversal in a mole crab *Emerita asiatica* (Abstract) *Inter. Symp. Invest. Reprod., Univ. Calif. Davis*, Aug. 27–31.

Subramoniam, T. 1979c. Heterosexual raping in the mole crab *Emerita asiatica. Int. J. Invertebr. Reprod.,* **1:** 197–199.

Touir, A. 1973. Influence de l'ablation des pédoucules oculaires su les glandes androgènes, les gonades, les caractères sexuels externes mâles et l'inversion sexuelle chez la Crevette hermaphrodite *Lysmata seticaudata* Risso. *C.R. Acad. Sci. Paris* **277:** 2541–2544.

Touir, A. 1977a. Données nouvelles concernant l'endocrinologie sexuelle des Crustacés décapodes *Natantia* hermaphrodites et gonochoriques. II. Maintien des gonies et évolution des gamétogenèses *in vivo* et *in vitro. C.R. Acad. Sci. Paris* **284:** 2515–2518.

Touir, A. 1977b. Données nouvelles concernant l'endocrinologie sexuelle des Crustacés Decapodes *Natantia* hermaphrodites et gonochoriques. III. Mise en évidence d'un contrôle neurohormonal du maintien de l'appareil génital mâle et des glandes androgènes exercé par le protocérébron médian. *C.R. Acad. Sci. Paris* **285:** 539–542.

TOUIR, A., 1977c. Données nouvelles concernant l'endocrinologie sexuelle des crustacés décapodes *Natantia* hermaphrodites et gonochoriques. I. Maintien des glandes and rogènes et role de ces glandes dans le controle des gamétogénèses et des caractères sexuels externes males. *Bull. Soc. Zool. Fr.* **102:** 375–400.

VARADARAJAN, S., AND SUBRAMONIAM, T. 1980. Histochemical investigation on vitellogenesis in the hermit crab *Clibanarius clibanarius*. *Int. J. Invert. Reprod.* **2:** 47–58.

WHARTON, G. W. 1942. A typical sand beach animal, the mole crab *Emerita talpoida* (Say) In: Ecology of sand beaches at Beaufort N. C. *Ecol. Monogr.* **12:** 137–181.

WENNER, A. M. 1972. Sex ratio as a function of size in marine crustacea. *Ann. Nat.* **10:** 321–330.

WITSCHI, E. 1971. Mechanism of sexual differentiation. Pp. 601–618 in M. Hamburgh and E. J. W. Barrington, Eds., *Hormones in Development*. Appleton, New York.

YALDWYN, J. C. 1966. Protandrous hermaphroditism in decapod prawns of the families Hippolytidae and Campylonotidae. *Nature* **209:** 1366.

Reference: *Biol. Bull.*, **160:** 175–188. (February, 1981)

HYDRA VIRIDIS: TRANSFER OF METABOLITES BETWEEN *HYDRA* AND SYMBIOTIC ALGAE

GLYNE THORINGTON AND LYNN MARGULIS

Department of Biology, Boston University, Boston, MA 02215

ABSTRACT

"Back transfer" of metabolites from food to endosymbiotic algae in the digestive cells of *Hydra viridis* was demonstrated. Brine shrimp nauplii labeled with tritiated precursors of protein and nucleic acids (DNA and RNA) were fed to light and dark grown hydras. The fate of the label after a single feeding with radioactive material in hydra and algal fractions was followed by scintillation counting and autoradiographic techniques. Labeled thymidine was incorporated into DNA in both light- and dark-grown hydras. Although the symbiosis persists indefinitely in hydras in darkness (7–10 days) the number of algae per cell is reduced.

Tritiated orotic acid and tritiated uridine, RNA precursors, were incorporated into the RNA fraction of hydra cells and algae. Tritiated leucine was incorporated into peptides and proteins, and to a lesser extent into simple sugars, oligosaccharides, and oligonucleotides in hydra and algal fractions. Thus the metabolites of the brine shrimp food are available to both partners.

A decrease over time in label introduced as ^{3}H-orotic acid and ^{3}H-uridine and incorporated into hydra RNA is compensated for by an increase in label in the algae, implying competition for constant quantities of metabolites from the single feeding. Although food availability, light, number of algae per cell, and other factors influence the quantity and rate of nutrient transfer between the partners, in both light and dark grown hydras the amount of "back transfer" of metabolites to the symbiotic algae is impressive.

INTRODUCTION

Green hydras, first reported in the 18th century, have been studied continually since Geddes (1882). All the many hydra strains isolated harbor characteristic algae of the genus *Chlorella*. These green hydras, *Hydra viridis* (synonyms in the literature include *Chlorohydra viridis* and *Hydra viridissima*), taken from freshwater ponds and streams all over the world, are easily maintained in the laboratory. They are kept in the light, fed crustaceans such as brine shrimp every 2 or 3 days, and must be carefully cleaned to prevent overgrowth of bacteria. Because the hydra is disassociable from its *Chlorella* sp. symbionts, *H. viridis* is a preferred laboratory organism for research on metabolic and growth relationships in symbiosis (Muscatine, 1967; Muscatine and Lenhoff, 1965a, b; Cook, 1972; Pardy, 1974). As in other invertebrate-algal endosymbioses, the algae, restricted to the gastrodermal (endodermal) cells of the hydras, transfer photosynthate to their hosts (Muscatine and Hand, 1958; Goreau and Goreau, 1960; Lenhoff and Muscatine, 1963). White, aposymbiotic hydras, rare or unknown in nature, occasionally have been observed in recently hatched hydras but can be produced routinely by treatment with high

Received 16 October 1980, accepted 1 December 1980.

light intensities in the presence of chemical inhibition of photosynthesis (Pardy, 1976).

A large volume of literature of the past decade deals with the metabolic relationships between freshwater hydras and their algal symbionts, with emphasis on the benefits to the host (Muscatine and Lenhoff, 1965b; Muscatine, 1967; Cernichiari *et al.*, 1969; Pardy and White, 1977). Most of the biochemical studies have been concerned with unidirectional movement of metabolites from autotroph to heterotroph. For example, in green hydras allowed to incorporate $^{14}CO_2$ photosynthetically (Muscatine and Lenhoff, 1965b) the algae-free ectoderm accumulated approximately 10–12% of the total fixed ^{14}C at a rather constant rate over 48 h. The specific activity of the ectodermal tissues from the green hydras was much higher than that of the aposymbiotic controls, suggesting a net translocation of fixed radioactive carbon to the host ectoderm.

Isolated symbiotic algae release about 85% of total fixed carbon, mainly as the disaccharide maltose (Muscatine, 1965). The nature of the algal metabolite excreted is influenced by the pH of the medium: Maltose is the primary product excreted at pH 4.5, but it decreases as the medium becomes more alkaline, with little or no maltose released at neutral pH (Cernichiari *et al.*, 1969). Traces of glucose, alanine, glycolic acid, and an unknown oligosaccharide also are excreted. Maltose thus is thought to be the major nutrient released to the host. The ^{14}C translocated to the animal host is detected in the animal tissue as small molecules, nucleic acids, alcohol soluble peptides, proteins, and glycogen (Roffman and Lenhoff, 1969). About 35% of the labeled carbon is present in hydra tissue as glycogen after 4 h incubation.

This movement of photosynthate seems to be of great survival value to hydras when food is limited. As long as illumination is adequate, starved green hydras survive for months, far longer than their aposymbiotic counterparts (Muscatine and Lenhoff, 1965a, b; and unpublished observations of ours and D. C. Smith, Oxford University). The algae's contribution to hydra growth efficiency (percent of energy, consumed as food, converted to new protoplasm; Slobodkin, 1962) increases in starved animals (Stiven, 1965). Although algae probably contribute organic materials that enhance the host's growth and survival, the algae probably do not affect growth by producing oxygen or removing carbon dioxide, since aposymbiotic hydras do not need algae when food is available.

The first sign of a reverse flow of metabolites was obtained when ^{14}C labeled brine shrimp were fed to hydras (Cook, 1972) in either constant darkness or constant light. After 48 h in the light, from 22% to 26% of the recovered activity was in the algal fraction, whereas in the dark 25% to 34% was in the algal fraction. Thus, in both light and darkness, chlorellae take up carbon from the hydra's ingested food. Furthermore, nutrients move from hydras to algae, because algae are retained in well-fed hydras kept in continuous darkness.

To verify further a bidirectional flow of nutrients, Cook (1972) fed ^{35}S-labeled food to *Aiptasia* sp., and separated the algal fraction from the host tissue. He found that algae take up and incorporate radioactive label into protein rather quickly, and retain about 15–30% of the label for at least three to five days.

The experiments reported here were designed to determine the nature of "back transfer" and to verify that it occurs regularly. Hydras were fed once with labeled brine shrimp and the fate of the label monitored in animal and algal fractions by scintillation counting. To locate the label, hydra cells and isolated algae were studied by autoradiography using tritiated precursors of protein, DNA, and RNA.

A large number of gram-negative rod-shaped bacteria also have been found in symbiotic relationship with green hydras (Margulis *et al.*, 1978; Wilkerson, 1980). In the Ohio hydra strain, used in most of the present studies, the bacteria are in vacuoles of the same gastrodermal cells in which the *Chlorella* reside. The bacteria have been cultivated in pure culture and identified as members of the species *Aeromonas punctata* (Berger *et al.*, 1979). The aeromonads are closely associated with the algal symbionts throughout the symbionts' life cycle (Thorington, 1980). However, intracellular bacteria are absent in the Carolina strain, and English hydras have as-yet-unidentified bacteria at the junctions of the ectodermal cells. Wilkerson (1980) verified that bacteria influence the metabolic activities of the host. She discovered that green hydras kept bacteria-free by antibiotic treatment take up 55% less labeled orthophosphate than green hydra controls with a normal complement of symbiotic bacteria. Our hydras were not treated to remove bacterial symbionts; however, the bacteria were about equally distributed between the animal and algal fractions (light microscopic observations). Hence their quantitative role was ignored.

MATERIALS AND METHODS

Collection and maintenance of hydras

Two different strains of *H. viridis* were used. The Carolina strain, obtained from the Carolina Supply Co., was used in only one early set of experiments. Because of their large size and sturdiness in culture, all later experiments were done on Ohio strain animals (a gift from Dr. C. Cook, Ohio State University).

Hydras were grown in mass cultures according to the methods of Lenhoff and Brown (1970). Before experiments, all hydras were cultured in "M" solution (Muscatine and Lenhoff, 1965a) at 20° ± 1°C, kept under constant illumination, and fed every other day to repletion on freshly hatched *Artemia* nauplii.

Experimental animals

Only hydras bearing one bud (hydranth) were used for experiments. They were placed in finger bowls and starved for 2 days before experimentation. Sixty hydras were used for incorporation studies involving precursors of protein and RNA. Since H^3-thymidine uptake into DNA is less than into RNA and protein precursors, 210 hydras were used for DNA experiments. During continuous-light experiments, the hydras were placed in an incubator at 20° ± 1°C, with light supplied by two cool white fluorescent bulbs 4–6 in from the hydras. For dark experiments, the finger bowls were wrapped in aluminum foil, placed in a cupboard at 22° ± 2°C, and exposed to no more than 2 min of light during feeding or cleaning.

Incubation of brine shrimp with radioisotopes

Freshly hatched *Artemia* nauplii were placed in small bowls containing 50 ml filtered seawater. Bacterial contamination was reduced by adding 50 mg streptomycin and 165,000 units of penicillin (Sigma Biochemical Co.). To this incubation medium, we added ^{3}H-leucine (specific activity, 60 Ci/m*M*), ^{3}H-thymidine (specific activity, 60.4 Ci/m*M*), ^{3}H-uridine (specific activity, 24.2 Ci/m*M*) or ^{3}H-orotic acid (specific activity, 11.1 Ci/m*M*). The final concentration of radioactivity in the medium in each case was: leucine 0.7 μCi/ml, thymidine 0.4 μCi/ml, uridine 1.0 μCi/ml, and orotic acid 0.3 μCi/ml. After 48 h incubation the nauplii were collected and thoroughly washed with "M" solution. They were then fed to the experimental

1 ml for 100 ml [illegible]

10 μCi = 1 ml

hydras. The culture solution was changed after one h, again at six h, and, for longer experiments, daily to lower microbial contamination.

Separation of algae from animal tissue

At intervals after feeding, hydras were removed, washed in ice-cold "M" solution, and homogenized in a glass tissue homogenizer. The suspension was then centrifuged at 300 rpm for 5 min in an International Clinical Centrifuge. The cloudly white residue containing the animal tissue was removed and stored. The green algal pellet (Muscatine, 1965b) was washed several times with "M" solution until a clear residue was obtained. The washings were added to the original animal fraction to give a total volume of 3 ml. A smear of the algal pellet on a slide showed minimal animal-tissue contamination. The algal cells were broken by sonication in ice-cold "M" solution for 2 min (30 s of sonication at 1500 cycles followed by 30 s of cooling, twice). The sonicate was brought to a total volume of 3 ml.

Assay of tritiated leucine into protein

The disrupted tissue, hydra and algae, was brought to a final volume of 3 ml by adding "M" solution. From this, 0.4 ml samples were removed for assay. To 1 ml of the suspension we added 1 ml of ice-cold 10% trichloroacetic acid (TCA). This stood at room temperature for about 15 min. A second 1 ml portion was similarly treated with hot 10% TCA and then placed in a 90°C water bath for 30 min. The TCA-soluble fractions were separated from the TCA-insoluble fraction by filtering the suspension through a 0.45 μm (pore size) Millipore filter. The soluble fractions were collected in a clean test tube. A 0.2 ml portion was removed from the soluble fractions for counting of radioactivity. The insoluble fractions were washed three times with 5% TCA before the filter paper discs were air-dried and then counted.

To 0.6 ml of the cold TCA-soluble fraction we added 3.2 ml of 95% ethanol. This was heated at 45°C for 45 min. Three ml of this suspension was filtered as above to obtain TCA-alcohol soluble and insoluble fractions. From the TCA-alcohol soluble fraction, 1 ml portions were used for radioactive counting. The TCA-alcohol insoluble fractions were washed several times with 80% ethanol before counting.

We added 3.2 ml of 95% ethanol to 0.6 ml of the original suspension. This was then incubated at 45°C for 45 min. Alcohol-soluble and insoluble fractions were obtained by filtering (0.45 μm/Millipore) 3 ml of the suspension. One-ml samples of the alcohol soluble fraction were used for radioactive counting. Alcohol insoluble fractions were washed with 80% ethanol before counting.

In these experiments the distribution of radioactivity was recorded as follows: TCA-soluble, ethanol-soluble fraction contains small molecules such as amino acids and monosaccharides; TCA-soluble, ethanol-insoluble fraction contains oligosaccharides and oligonucleotides; TCA-insoluble, ethanol-soluble fraction contains lipid and lipid soluble components, most small proteins and peptides; and finally TCA-ethanol and hot TCA-insoluble fraction contains the rest of the proteins.

Extraction and assay of radioactivity in RNA

RNA was extracted from the animal and algal fractions by the hot-phenol extraction method of Scherer and Darnell (1962). Distilled phenol was equilibrated with distilled water overnight to give a final concentration of approximately 87% (v/v). One ml of this solution was added to 1 ml of homogenate (hydra fraction) or sonicate (algal fraction) containing 200 mg of yeast RNA (Sigma Biochemical Co.). After vigorous shaking, the mixture was incubated at 65°C for 10 min, cooled with tap water, and centrifuged at 600 *g* for 5 min at room temperature. The lighter aqueous phase was removed with a Pasteur pipette and an equal volume of phenol solution added. We repeated this extraction sequence three times. After the final centrifugation, two volumes of cold 95% ethanol (pre-chilled at −17°C) were added to the aqueous phase. After quick mixing the solution was kept at −17°C overnight to let RNA precipitate.

On the following day, the material was centrifuged at 7000 *g* for 10 min at 4°C. The ethanol was decanted and the RNA pellet resuspended in 4 ml ethanol. The washing sequence was repeated until we detected no phenol odor. The pellet resuspended in 2 ml 0.01 *M* Tris-HCl buffer, pH 7.4, containing 0.01 *M* NaCl and 0.02 *M* $MgCl_2$. This solution was centrifuged at 10,000 *g* for 15 min, and the supernatant retained to assay radioactivity in RNA.

Tritiated RNA was measured by the disc method (Yu and Feigelson, 1971). We applied 200 ml portions of RNA to filter paper discs (Whatman 3MM, 2.3 cm diameter), air dried the discs, and held them in freshly prepared 10% TCA at 0°C for 3 h to "fix" the sample. The 10% TCA then was decanted and the discs were washed three times with 5% TCA at 0°C to remove acid soluble radioactivity. They were then divided into two groups, of two sample discs and two blanks each. One group was left in cold 5% TCA solution and the other incubated for 20 min at 90°C. All discs were then washed three times with cold 5% TCA, twice extracted with ethanol:ether 1:1 (v/v) at 37°C, and dried under a lamp. Radioactivities of the discs were determined by counting in 10 ml liquid scintillation fluid in a Packard Tricarb Counter.

Enzyme treatment was performed to verify that radioactivity was actually in RNA. The extraction procedures were repeated but to the nucleic acid precipitate was added McIlvaine's citrate phosphate buffer containing RNase (Sigma Biochemical Co.) to give a final concentration of 1 mg/ml at pH 6.7. The sample was incubated at 37°C for 2 h. After incubation the procedure continued as outlined above.

Extraction and assay of radioactivity in DNA

DNA was extracted from the animal and algal fractions by the following method: to 2 ml of homogenate or sonicate containing 200 mg of salmon DNA-carrier was added 1 ml cold 0.6 *N* perchloric ($HClO_4$) acid. This suspension was placed in an ice bath for 10 min and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was discarded. The pellet was washed twice with 5 ml cold 0.2 *N* perchloric acid. The washes were decanted and the tube allowed to drain onto filter paper for a few minutes. The pellet, with 4 ml of 0.3 *N* potassium hydroxide added, was incubated in a shaking water bath at 37°C for 1 h, and then chilled in an ice bath for 15 min. We then added 0.6 *N* perchloric acid and centrifuged the tubes at 10,000 rpm for 10 min at 4°C. This supernatant was retained. The pellet was washed twice with 5 ml of 0.2 *N* perchloric acid. After another centrifugation as above, the supernatant was retained. The three supernatants were combined and brought to a final volume of 50 ml with distilled water. This fraction contained the RNA. The precipitate was dissolved in 5 ml of 0.3 *N* potassium hydroxide, incubated in a shaking water bath overnight, and the next day diluted to 15 ml with distilled water to obtain the DNA fraction.

To verify that the radioactivity was actually in DNA, the procedure was repeated but the precipitates incubated in buffered DNase solutions (Sigma Biochemical Co., 1 mg/ml) for 2 h at 37°C. Equal portions were counted in a toluene-based liquid scintillation fluid.

Autoradiographic experiments

To study the incorporation of label into nucleic acids (RNA and DNA) in the algae of *Hydra viridis*, labeled hydras were washed 2–3 times with large volumes of cold "M" solution. The algae were separated from the animal fractions as described above, and the algal pellet further washed several times to remove any exogenous radioactivity. To 0.2 ml algal medium we added 0.2 ml of 6% acetic acid to fix the cells. Algae then were smeared on acid-washed albuminized slides and fixative allowed to evaporate. The slides were washed in distilled water, air dried, and stored at 4°C until they were autoradiographed with Kodak AR 10 stripping film. Test exposure of autoradiographed RNA slides was 21 days, and for DNA 93 days. Some slides were reserved for nuclease treatment before autoradiography. For DNA, spleen DNase II (Sigma Chemical Co., St. Louis Mo.) 1 mg/ml, buffered in McIlvaine's phosphate buffer at pH 6.8–6.9 in 0.002 *N* $MgSO_4$, was incubated at 37°C for 4 h. For RNA, RNase (grade A from bovine pancreas) 1 mg/ml in McIlvaine's phosphate buffer, pH 6.7, was incubated at 37°C for 4 h. All slides were washed before being autoradiographed.

For incorporation of tritiated leucine, after several washes a 0.5 ml volume of animals was placed in 0.5 ml maceration solution (1:1:13 v/v/v glycerol:glacial acetic acid:distilled water) and fixed in this solution for 1 h. After fixation, a smear of the different cell types was air dried and autoradiographed. Exposure time was 21 days.

All autoradiographs were developed for 5 min in Kodak D-19 developer at 10–16°C, rinsed in Kodak stop bath, and then placed in Kodak rapid fix for 3 min. The slides were then rinsed in running water for 30 min. Algae grains were then counted by white light and phase contrast microscopy.

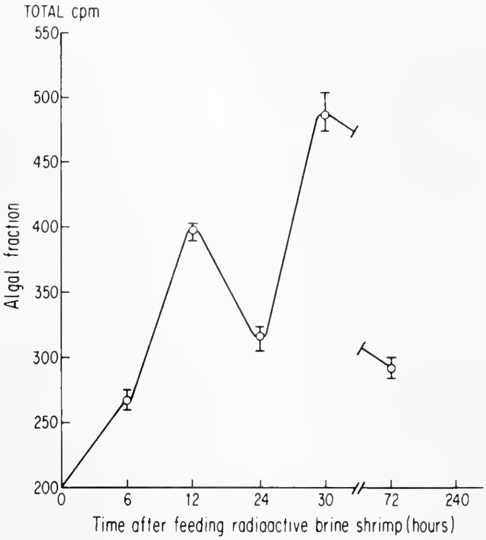
RESULTS

Uptake of tritiated leucine by algae

Figure 1 shows the results of experiments in which algae were removed from hydras kept in the light and dark. The quantity of label in the algae under light and dark conditions fluctuated. Algae in hydras incubated in the dark took up more label than those in the light. However, due to inactivity of the algae, the label was not as efficiently used as in the light. Leakage or expulsion of algae could be responsible for the drastic drops at 72 and 240 h. In the light experiments, there were peaks at 12 h and again at 30 h. These might reflect times of increased algal activity. After 10 days in the dark, the algae still contained label.

Distribution of label in major fractions

Figure 2 (left) shows that most of the label in algae in hydras kept under constant light conditions was found in the small-molecule and protein fractions. Label in these fractions fluctuated very little over 72 h. The TCA soluble alcohol-insoluble labeled fraction (oligosaccharides, oligonucleotides) might represent intermediate metabolites. The trace amounts of label introduced as leucine in the nucleic acid fraction might be contamination from other fractions.



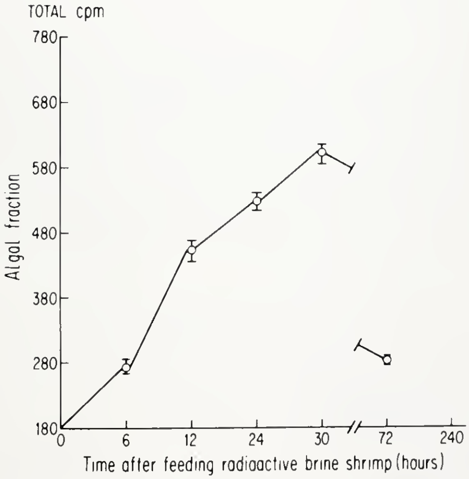


FIGURE 1. Uptake of tritiated leucine in algal fractions of light-grown hydras (above) and dark-grown hydras (below). Vertical bars show standard deviation. Each point shows means of total radioactivity counts of samples from four independent experiments, each using 60 hydras.

In the dark, label in the three major fractions just mentioned fluctuates more (Fig. 2, right). During the first six h, approximately 58% of the label was in the TCA-alcohol soluble fraction and about twice as much label was in the TCA-soluble-alcohol-insoluble fraction. Over the next 18 h, the radioactive material slowly changed, moving from the TCA-alcohol-soluble to the TCA-insoluble-fraction, indicating incorporation of amino acids into proteins. This continued about 30 h, after which the relative amounts of radioactivity in the small-molecule and protein fractions remained constant.

Neither in the light nor in the dark were lipid or lipid soluble compounds labeled, primarily because no lipid precursors were utilized. Since label introduced

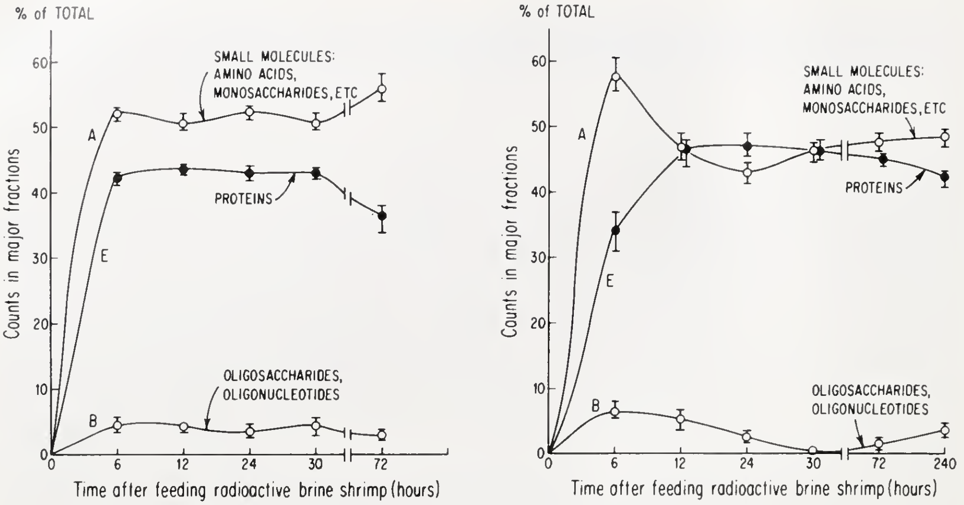


FIGURE 2. Tritiated leucine in major fractions of algae from light-grown (left) and dark-grown (right) hydras. Vertical bars show standard deviation. Each point shows the mean from four independent experiments, each using 60 hydras.

as leucine was seen in other fractions besides amino acids and proteins, it was being assimilated into algal metabolites. In the animal fraction from dark grown hydras at around 30 h label was incorporated into the TCA-insoluble alcohol-soluble lipid-soluble (lipid and lipid-soluble compounds; small protein) fraction. With longer time intervals, these compounds were lost either as a result of leakage or due to utilization by the organism. The ^{3}H-leucine data is summarized in Table 1. Figure 3 shows the distribution of label among the major biochemical fractions in the light- and dark-grown animal fractions.

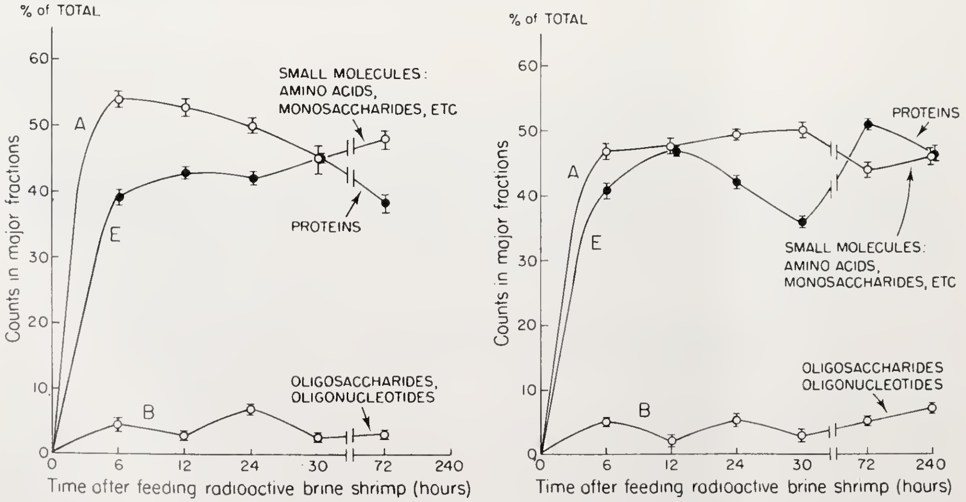


FIGURE 3. Tritiated leucine in major fractions from animal fraction from light-grown (left) and dark-grown (right) hydras. Vertical bars show the standard deviation. Each point is mean from four independent experiments, each using 60 hydras.

TABLE I

Algal and animal fraction ^{3}H-Leucine. Mean ± SD.

Fraction	6 h	12 h	24 h	30 h	72 h	10 days
Agal, light						
Amino acids & sugars	52 ± 0.64	50.8 ± 0.92	52.6 ± 1.14	50.2 ± 0.89	52.6 ± 0.89	—
Oligosaccharides & oligonucleotides	4.6 ± 0.86	4.5 ± 0.165	4.2 ± 1.15	4.8 ± 1.02	3.2 ± 0.70	—
Lipids	—	—	—	—	—	—
Nucleic acids	2.0 ± 0.96	1.16 ± 0.70	1.7 ± 0.96	1.8 ± 1.11	4.2 ± 1.19	—
Proteins	41.2 ± 0.90	43.4 ± 0.43	42.8 ± 1.3	42.7 ± 0.73	39.65 ± 1.95	—
Algal, dark						
Amino acids & sugars	57.8 ± 2.7	47 ± 2.0	42.9 ± 1.57	46.2 ± 1.42	48 ± 1.63	48.5 ± 1.02
Oligosaccharides & oligonucleotides	6.9 ± 1.48	5.2 ± 1.45	2.7 ± 0.58	0.53 ± 0.56	1.6 ± 0.70	3.8 ± 0.75
Lipids	—	—	—	—	—	—
Nucleic acids	0.76 ± 0.79	0.86 ± 0.84	7.36 ± 0.89	6.7 ± 1.15	4.9 ± 1.20	4.5 ± 0.56
Proteins	34.16 ± 2.9	46.5 ± 2.0	47.16 ± 1.67	46.2 ± 1.34	45.0 ± 0.75	42.5 ± 0.59
Animal, light						
Amino acids & sugars	54 ± 1.608	53.6 ± 1.34	50.3 ± 1.22	45.6 ± 2.29	48 ± 1.13	—
Oligosaccharides & oligonucleotides	4.4 ± 1.17	3.5 ± 1.12	6.7 ± 1.19	2.4 ± 0.31	3.3 ± 0.84	—
Lipids	—	—	—	—	—	—
Nucleic acids	1.8 ± 1.25	—	0.5 ± 0.53	5.9 ± 2.19	10.32 ± 1.05	—
Proteins	39 ± 1.50	42.7 ± 1.04	41.9 ± 1.69	45.5 ± 1.04	38.25 ± 1.41	—
Animal, dark						
Amino acids & sugars	46.6 ± 1.49	47.6 ± 0.33	48.9 ± 1.81	50 ± 1.73	44 ± 1.45	45.9 ± 0.27
Oligosaccharides & oligonucleotides	5.2 ± 1.19	2.0 ± 1.07	5.7 ± 1.42	3.4 ± 0.85	5.0 ± 0.53	7.1 ± 0.79
Lipids	0.3 ± 0.52	—	—	5.6 ± 1.55	—	—
Nucleic acids	6.6 ± 1.72	3.5 ± 1.44	5.2 ± 1.65	5.1 ± 1.45	0.2 ± 0.32	0.2 ± 0.38
Proteins	41 ± 1.08	46.6 ± 1.79	40 ± 1.47	35.6 ± 1.82	50.5 ± 1.57	46.4 ± 0.48

RNA precursors into algal RNA

Tritiated orotic acid and tritiated uridine, both RNA precursors, were taken up and incorporated into both animal and algal fractions (Fig. 4). Uptake of RNA precursors differed in algae obtained from light- and dark-grown animals (Fig. 4). In the algal fraction from light-grown hydras, less label delivered as H^3 uridine was incorporated into nucleic acid relative to label delivered as ^{3}H-orotic acid. However, after 30 h with both precursors about 86–89% of the label was in nucleic acid. Therefore, the ^{3}H-uridine is apparently accumulated by the algae but less readily incorporated into nucleic acid in the light than ^{3}H-orotic acid.

Slightly more ^{3}H-orotic acid was incorporated into nucleic acid, compared to the labeled uridine, in dark-grown hydras (Fig. 5). RNA precursors in algal macromolecules continued to increase for 30 h in the light and dark. So many variables affect these results that quantitative and direct comparison of precursors is inappropriate. For example, environmental conditions (such as temperature) and probably the rate at which various tissues used label varied.

Treatment with bovine pancreatic RNase reduced the total counts to background values, verifying that most of the label introduced as orotic acid or uridine was incorporated into RNA.

DNA precursors into algal DNA

The uptake of ^{3}H-thymidine in the insoluble fraction of the algae is shown in Figure 6. In light-grown hydras the algae are very active. They divide at a rate

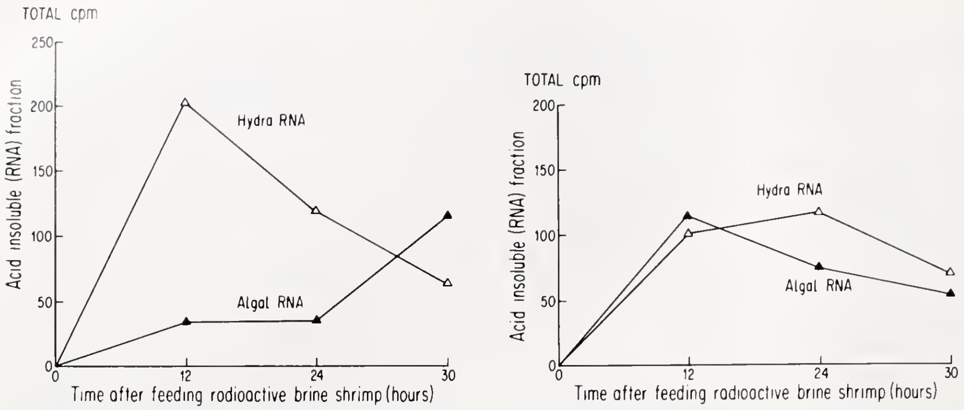
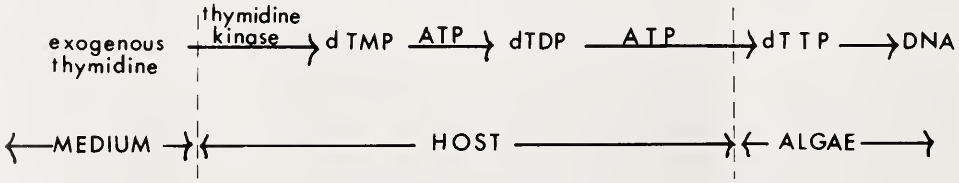


FIGURE 4. Tritiated uridine uptake and incorporation into RNA of animal and algal fractions from light-grown (left) and dark-grown (right) hydras. Each point is mean from four independent experiments, each using 60 hydras.

similar to that of cells of their hosts. The doubling time of green hydras is 2.0 days (Cook and Rupright, 1980). There was no statistically significant difference between the amounts of label incorporated into algae from light-grown and dark-grown hydras. With prolonged exposure, the quantity of labeled DNA decreased because of cell division.

According to Wanka *et al.* (1970), chlorellae lack the enzyme thymidine kinase. They take up, but degrade, the deoxynucleoside, and do not incorporate thymidine into DNA. In fact chlorellae utilize exogenous uridine more readily than thymidine as a precursor for DNA synthesis. They probably synthesize DNA by means of thymidylate synthetase. The symbiotic algal incorporation of thymidine into DNA seems to imply that the host kinases provide triphosphates which can then be utilized as well by the endosymbiotic chlorellae. Incorporation of thymidine into symbiotic algae DNA may be as indicated in this scheme:



Radioautography

Figure 7 shows a representative radioautograph, of algae isolated from light-grown hydras after 24 h in ^{3}H-leucine. The label was distributed over the isolated algae and the animal tissue.

Treatment of the cells with deoxyribonuclease and ribonuclease before autoradiography significantly decreased the amount of label, indicating that the labeling was due to incorporation into DNA or RNA respectively.

DISCUSSION

Cook (1972) suggested hydra "back transfer" of photosynthate to algae. Not only is carbon directly introduced as translocated bicarbonate across the vacuolar

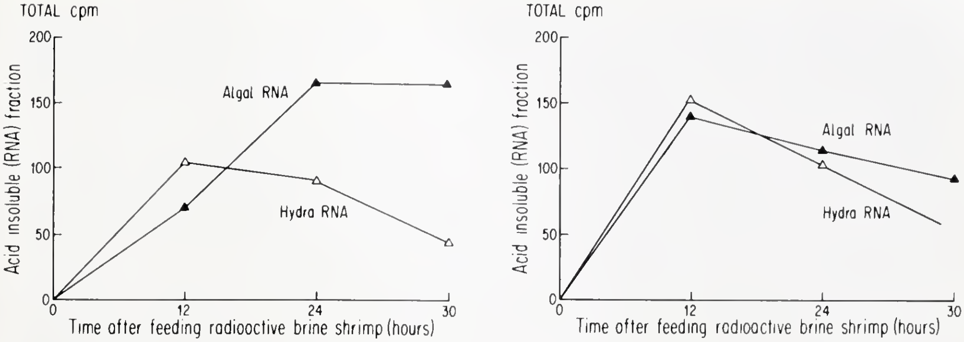
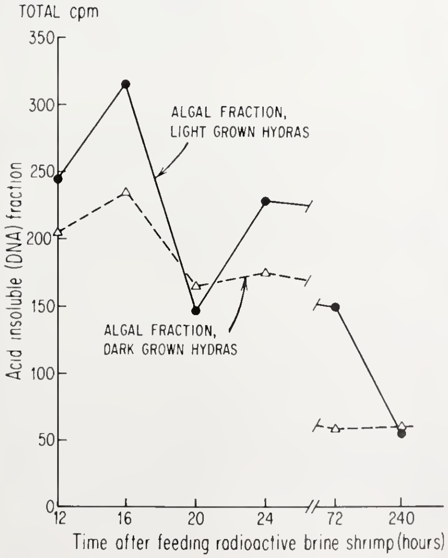


FIGURE 5. Tritiated orotic acid uptake and incorporation into RNA of animal and algal fractions from light-grown (left) and dark-grown (right) hydras. Each point is mean from four independent experiments, each using 60 hydras.

membranes of the symbionts, but protein precursors, nucleotide triphosphates, and ribonucleic acid precursors are also translocated as such. Labeled amino acid assimilated readily into algal and hydra proteins. It was also found in small molecules, such as amino acids, monosaccharides, oligosaccharides, and oligonucleotides. In hydras grown under constant light, there was little fluctuation in the small-molecule fraction, which incorporated most (about 52%) of the label. However, in dark-grown hydras, there was a correlation between the amount of label in the TCA-alcohol and soluble insoluble fractions. Amino acid incorporation into algae protein occurred, but with time hydrolysis of protein occurred as well. That is, the decrease



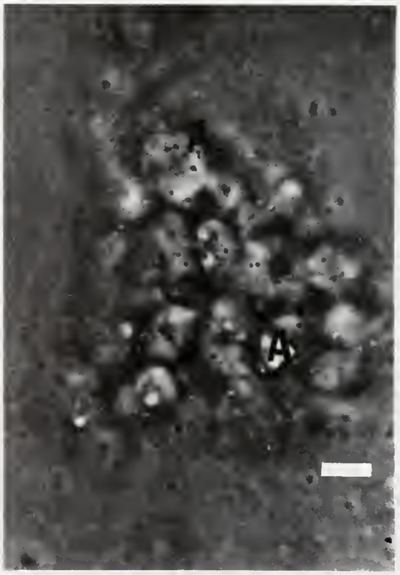


FIGURE 6. (Left) Tritiated thymidine uptake and incorporation into animal and algal fractions from light- and dark-grown hydras. Each point is mean from three independent experiments, each using 120 hydras.

FIGURE 7. (Right) Radioautograph of tritiated leucine labeled algae (A). Light micrograph (bar = 10 μm).

in label in the protein after 30 h corresponding to a rise in the other two fractions may be due to degradation of protein to supply energy for survival. The fractionation procedure did not elucidate whether the increase in the small molecule fraction reflected an increase in amino acids or in sugars.

Since photosynthesis was not occurring in the dark, and label appeared in the algal fractions, exogenous food (fed as brine shrimp to the host) was being hydrolyzed and taken up by the symbionts. The algae population in the gastrodermal cells decreased slightly after 10 days in darkness, from about 16 to about 11 algae per digestive cell. This suggests that on a per-cell basis, there was more incorporation in hydras maintained in the dark than in the light.

The differences in distribution of label between algae obtained from light- and dark-treated animals may reflect differences in the rate of assimilation of label or qualitative differences in metabolism of algae kept under these different conditions.

The conversion of label from the acid-soluble to the acid-insoluble fractions implied protein synthesis. The rate of conversion over 72 h in the animal fraction was correlated with that in the algal fraction in hydras incubated in the light. After 72 h, in both animal and algal light fractions, 40% of the label was in the form of protein. The correlation was less in dark-grown hydras.

Green hydras starved in the light derive about 75% of their energy from carbohydrates (Pardy and White, 1977), mainly by algal photosynthesis. Since in the present experiments the animals were fed only once, over the longer intervals used starvation probably set in. Although the animal's main energy source then would be photosynthetic products, some of the labeled products also may have been shunted into the carbohydrate metabolic pathway, thus supplying some of the host's metabolic needs (Fig. 3).

Thymidine was taken up by algal symbionts in both light- and dark-grown hydras. The total amount of label in both algal and host fractions is about twice as great in hydras incubated in the light than in the dark. The proportion of label in the animal fraction relative to the algal fraction remained high in hydras incubated in the light for 2 days, but by 3 days it decreased, presumably because of increased relative rate of host cell division.

Pardy (1974) reported a slight transient increase in growth rate of algae in hydra kept in the dark. Our studies showed increased uptake of thymidine in the algal fraction of dark-grown hydras at 16 h, probably reflecting algal growth. This was followed by a sharp decline in activity over the remaining 9½ days.

In the dark, algae may compete among themselves and with their hosts for metabolites (Pardy, 1974). This may slow hydra growth as much as 20%, and algae growth even more. Our observations seem consistent with Pardy's conclusions. Metabolites of brine shrimp (food) origin are available to both partners. A decrease in label in animal with time is compensated for by an increase of label in the algae. This is especially evident in ^{3}H-orotic acid and ^{3}H-uridine incorporation into RNA. Thus, host and algae may compete for constant quantities of metabolites from one feeding. Since we found no separate algal nucleotide pool, the algae probably have access to the hosts' ribonucleotide pool. In the dark, host cells contain more label relative to the algae cells. In fact, with prolonged darkness (7–10 days), the number of algae per cell is reduced. But in fed hydras, the symbiosis apparently persists indefinitely—or at least 3–4 months, until the experiment ended. Food availability and light influence the quantity and rate of nutrient transfer as well as the ratio of partner cells in this symbiosis.

As the label in the thoroughly washed algal pellet was in the algal fraction, and this was not due to contamination with animal tissue, "back transfer" of

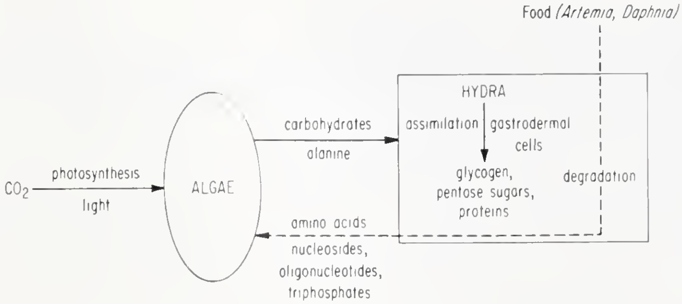


FIGURE 8. Nutrient exchange between hydra and symbiotic algae.

metabolites from the host to the algae occurs. From this source, dark-grown chlorellae obtain nutrients to metabolize and grow. Even in the light, large quantities of small food molecules are transferred to the autotrophs. Figure 8 summarizes the major routes of nutrient exchange between hydras and their symbiotic algae.

The bacterial symbionts of hydra probably also affect these metabolic interactions. Bacteria-free hydras double in 1.7 days as compared to the 2.0 days doubling time of green hydra with bacteria (Cook and Rupright, 1980). Our results and those of Wilkerson (1980) suggest that further quantitative studies of metabolic interactions in hydra symbioses must consider the roles of all three partners in nutrient flow: *Hydra viridis* (host), *Chlorella* sp. (symbiotic algae), and *Aeromonas punctata* (symbiotic bacteria) cells. The separability, manipulability, and experimentally achieved independent growth of the three partners in this symbiosis, coupled with the diverse quality and large quantity of metabolite flow, makes *Hydra viridis* and its microbes ideal for testing models of the evolutionary origin of symbioses (Smith, 1979).

ACKNOWLEDGMENTS

We thank Profs. David Smith and Clayton Cook, as well as Stephen Fracik for their suggestions, advice, and for our supply of hydras.

This work was supported by NASA NGR-004-025, a Grant-in-Aid of Research from Sigma Xi (to Glyne Thornington), Boston University Graduate School, and the Guggenheim Foundation (Lynn Margulis). We also acknowledge support by the California Institute of Technology, Division of Geological and Planetary Sciences.

LITERATURE CITED

BERGER, B., G. THORINGTON, AND L. MARGULIS. 1979. Two aeromonads: growth of symbionts from *Hydra viridis*. *Curr. Microbiol.* **38:** 5–10.

CERNICHIARI, E., L. MUSCATINE, AND C. C. SMITH. 1969. Maltose excretion by the symbiotic algae of *Hydra viridis*. *Proc. R. Soc. Lond. Ser. B. Biol. Sci.* **173:** 557–576.

COOK, C. B. 1971. Transfer of ^{35}S-labelled material from *Aiptasia* sp. to its endosymbiotic zooxanthellae. Pp. 218–224 in H. M. Lenhoff, L. Muscatine and L. V. Davis, Eds., *Experimental coelenterate biology*. University of Hawaii Press, Honolulu.

COOK, C. B. 1972. Benefit to symbiotic zoochlorella from feeding by green hydra. *Biol. Bull.* **142:** 236–242.

COOK, C. B., AND G. L. RUPRIGHT. 1980. The effect of bacterial endosymbionts on growth and survival in *Hydra viridis*. *Am. Zool.* **20:** 776.

GEDDES, P. 1882. The yellow cells of radiolarians and coelenterates. *Proc. R. Soc. of Edin. Ser. B. Biol. Sci.* **11:** 377–396.

GOREAU, T. F., AND N. I. GOREAU. 1960. Distribution of labelled carbon in reef-building corals with and without zooxanthellae. *Science* **131:** 668–669.

LENHOFF, H. M., AND B. ROFFMAN. 1971. Two methods for fractionating small amounts of radioactive tissue. Pp. 265–271 in H. M. Lenhoff, L. Muscatine and L. V. Davis, Eds., *Experimental coelenterate biology*. University of Hawaii Press, Honolulu.

LENHOFF, H., AND R. BROWN. 1970. Mass culture of hydra: an improved method and its application to other aquatic invertebrates. *Lab. Anim.* **4:** 139–154.

LENHOFF, H. M., AND L. MUSCATINE. 1963. Symbiosis: On the role of algae symbiotic with hydra. *Science* **142:** 956–958.

MARGULIS, L., G. THORINGTON, B. BERGER, AND J. STOLZ. 1978. Endosymbiotic bacteria associated with the intracellular green algae of *Hydra viridis*. *Curr. Microbiol.* **1:** 227–232.

MUSCATINE, L. 1967. Glycerol excretion by symbiotic algae from corals and *Tridacna* and its control by the host. *Science* **156:** 516–519.

MUSCATINE, L. 1971. Endosymbiosis of algae and coelenterates. Pp. 179–191 in H. M. Lenhoff, L. Muscatine and L. Davis, Eds., *Experimental Coelenterate Biology*. University of Hawaii Press, Honolulu.

MUSCATINE, L., AND C. HAND. 1958. Direct evidence for transfer of materials from symbiotic algae to the tissues of a coelenterate. *Proc. Nat. Acad. Sci. USA* **44:** 1259–1263.

MUSCATINE, L. 1965. Symbiosis of hydra and algae. III. Extracellular products of the algae. *Comp. Biochem. Physiol.* **16:** 77–92.

MUSCATINE, L., AND H. M. LENHOFF. 1965a. Symbiosis of hydra and algae. I. Effects of some environmental cations on growth of symbiotic and aposymbiotic hydra. *Biol. Bull.* **128:** 415–424.

MUSCATINE, L., AND H. M. LENHOFF. 1965b. Symbiosis of hydra and algae. II. Effects of limited food and starvation on growth of symbiotic and aposymbiotic hydra. *Biol. Bull.* **129:** 316–328.

PARDY, R. 1974. Some factors affecting the growth and distribution of the algal endosymbionts of *Hydra viridis*. *Biol. Bull.* **147:** 105–118.

PARDY, R. 1976. The production of aposymbiotic green hydra by the photodestruction of their symbiotic algae. *Biol. Bull.* **151:** 225–235.

PARDY, R., AND B. WHITE. 1977. Metabolic relationships between green hydra and its symbiotic algae. *Biol. Bull.* **153:** 228–236.

ROFFMAN, B., AND H. M. LENHOFF. 1969. Formation of polysaccharides by hydra from substrates produced by their endosymbiotic algae. *Nature* **221:** 381–382.

SCHERER, K., AND J. E. DARNELL. 1962. Sedimentation characteristics of rapidly labelled RNA from Heal Cells. *Biochem. Biophys. Res. Comm.* **7:** 486–490.

SLOBODKIN, L. B. 1962. Energy in animal ecology. Pp. 69–101 in J. B. Cragg, Ed., *Advances in ecological research*. Vol. I. Academic Press, New York.

SMITH, D. C. 1969. From extracellular to intracellular: The establishment of a symbiosis. *Proc. R. Soc. London Ser. B. Biol. Sci.* **204:** 115–130.

STIVEN, A. E. 1965. The relationship between size, budding rate, and growth efficiency in three species of hydra. *Res. Pop. Ecol.* **7:** 1–15.

THORINGTON, G. 1980. The algal and bacterial symbionts of *Hydra viridis*: Metabolic relations and transmission through the host sexual cycle. Ph.D. Dissertation. Boston University Graduate School, Boston, MA. 207 pp.

WANKA, F., H. F. JOOSTEN, W. J. DE CRIP. 1970. Composition of synthesis of DNA in synchronously growing cells of *Chlorella*. *Arch. Microbiol.* **75:** 25–36.

WILKERSON, F. 1980. Bacterial symbionts in green hydra and their effects on phosphate uptake. *Microbiol. Ecol.* **6:** 85–92.

YU, F., AND P. FIEGELSON. 1971. Paper disc estimation of radioactive RNA. *Analyt. Biochem.* **39:** 319–321.

Reference: *Biol. Bull.*, **160:** 189–197. (February, 1981)

INHALING WITHOUT RIBS: THE PROBLEM OF SUCTION IN SOFT-BODIED INVERTEBRATES

THOMAS G. WOLCOTT

Department of Marine Science and Engineering, North Carolina State University,
Raleigh, N. C. 27650

ABSTRACT

Most animals rely upon rigid skeletal structures to withstand the negative pressures required for inhalation. Some soft-bodied invertebrates (innkeeper worms, sea cucumbers) have pumping cloacae that are able to "inhale" without any hard supporting structures. Geometric analyses suggest that inhalation cannot be produced by relaxation of coelomic pressure and contraction of radial muscles connecting the body wall. Coelomic pressure must be maintained for body wall support and radial muscle antagonism. Mathematical models predict that maximum attainable suction in a hydrostatically supported sac-within-a-sac like the cloaca-body wall system will be proportional to coelomic pressure and exponentially related to the ratio of body wall diameter to cloacal diameter. A mechanical model gives results consistent with these predictions. "Cloacal" suctions are proportional to "coelomic" pressure, and maximum suction (20 × coelomic pressure) is produced when the cloaca is small relative to the body wall. *In vivo* recordings of pressure relationships during cloacal pumping in innkeeper worms (*Urechis caupo*) confirm that coelomic pressure is not relaxed to permit inhalation. No animals have been found which use a hydrostatically-supported system to produce extreme suction, with the possible exception of nematodes. Squids apparently utilize the potential of such a mechanism for producing large stroke volumes at low suctions with minimum structural complexity; the mantle is a sac-within-a-sac system which pumps large volumes for respiration and locomotion.

INTRODUCTION

Suction by animals is usually thought to require a rigid framework to support the negative pressure within the animal. The classic example is inhalation in vertebrates. Mammals can inhale because they have a rib cage and a diaphragm. Contraction of the domed diaphragm expands the thoracic cavity, creating negative pressure. The ribs prevent collapse of the walls, and the pressure is equalized by air being drawn into the lungs. A frog cannot inhale directly because it has no rib cage or diaphragm and therefore cannot generate negative pressure in the thoracic cavity. Instead it must use the buccal cavity, which does have a rigid framework and a movable floor, as a pump to inflate the lungs with positive pressure. It appears, then, that a rigid framework with movable elements is a *sine qua non* of suction in vertebrates.

Most sucking invertebrates conform to this generalization. Scorpions, spiders (Parry, 1954), and a few crabs that have been reported to produce suction (Wolcott, 1976; Greenaway & Taylor, 1976) are all arthropods, with rigid exoskeletons. Some invertebrates, though, manage to produce suction in the absence of hard parts.

Received 18 April 1980, accepted 2 October 1980.

Notable among these are the holothuroid echinoderms (sea cucumbers) and certain large echiurans (innkeeper worms). These soft-bodied animals use the cloaca as a pump to fill a "water lung" which meets a large proportion of their gas exchange requirements (Winterstein, 1909; Redfield & Florkin, 1931; Bertolini, 1933, Newell and Courtney, 1965).

These animals must produce negative internal pressures to draw in water, but they have no permanent rigid skeleton to support the body wall against collapse. Indeed, their hydrostatic skeleton only provides rigidity if positive internal pressures are maintained. This paper presents a theoretical solution to this apparent paradox and a mechanical model demonstrating the hydrostatics involved. It also compares the theoretical predictions and model performance to the functioning of living cloacal breathers, and comments on the implications for suction among other soft-bodied invertebrates.

Morphology of cloacal pumps

Cloacal breathers share several structural characteristics (Fig. 1). The body wall, although muscular, is intrinsically quite flexible; the animals become limp when dead or anesthetized. The thin-walled, contractile cloaca lies posteriorly in the capacious coelom, connected to the body wall with numerous radial muscles. Posteriorly it opens through the anus; anteriorly it communicates through sphincters with the hindgut and, in the case of sea cucumbers, with the respiratory trees.

A typical respiratory cycle in one of these cloacal breathers consists of three phases: inhalation, lung filling, and exhalation. The mechanisms of lung filling and exhalation are straightforward. Lung filling requires one (Pearse, 1908; Pantin & Sawaya, 1953) to twenty or more (Crozier, 1916; Ogawa, 1928; Tao, 1930) contractions of the cloaca with the anal sphincter closed and the sphincter into the respiratory structure open, forcing cloacal water into the lung. Exhalation involves opening both sets of sphincters and permitting coelomic pressure to collapse the flexible lung, expelling the contents to the exterior.

The mechanism by which the cloaca is refilled so that it may once again pump up the lung is not so obvious. Buddington (1937) hypothesized a two-step ". . . mechanism involved in expansion of the cloaca and thus in the respiratory intake of that organ: 1. General relaxation of the body-wall tissues, including muscles there present, with consequent release of pressure on all organs in the coelom. 2. Active contraction of the numerous separate muscle bundles leading from the cloacal to the body wall." Buddington conceded that his first step is ". . . hardly susceptible of proof on account of the numerous factors involved; but the validity of such assumptions can hardly be doubted since the situation is covered by applying the simplest principles of hydrodynamics." Other workers, although not directly addressing the inhalation question, seem to hold assumptions consistent with Buddington's.

The assumption that cloacal expansion is accomplished by contraction of the radial muscles and relaxation of the body wall presents some difficulties. The radial muscles are anchored to the body wall. Since the body wall is not inherently rigid, it will tend to collapse when the radial muscles contract, unless its distension is maintained by coelomic pressure. An increase in coelomic pressure to antagonize the tension exerted by the radial muscle will, however, increase the hydrostatic force tending to collapse the cloaca. This will require greater tension in the radial muscles, which would appear to require a further increase in coelomic pressure to prevent collapse of the body wall, and so forth. The cloacal breathers appear to be like the man attempting to lift himself by his bootstraps.

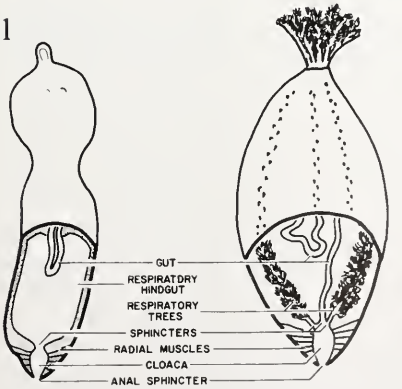
The solution to this apparent paradox lies in the geometry of the animals. The total force preventing collapse of the body wall around the cloaca is equal to the difference between coelomic and ambient pressure times the area of the body wall available for attachment of radial muscles, whereas the total force hindering expansion of the cloaca is the difference between cloacal and the coelomic pressure times the area of the cloaca. One would predict, then, that a net force would be available for expansion of the cloaca if the attachment area of radial muscles on the body wall exceeded the attachment area of those muscles on the cloacal wall. This is indeed the situation in cloacal breathers (Fig. 1), not surprisingly since the cloaca must be smaller in diameter than the body wall enclosing it.

MATERIALS AND METHODS

The maximum theoretical suction which could be developed by such a hydrostatically-supported "sac-within-a-sac" was calculated as a function of coelomic pressure and body geometry for two simplified body plans. A cylindrical body of indefinite length with a coaxial cloaca (Fig. 2), and a spherical body with a concentric cloaca (Fig. 3), were considered as limiting cases; the geometry of living cloacal breathers lies between these extremes. Several assumptions were made to simplify calculations. The body and cloacal walls were considered infinitely compliant, and effects of tension in the walls therefore disregarded. Static equilibrium was assumed, avoiding frictional and other hydrodynamic effects and allowing the sum of forces across any plane of section to be set at zero.

To test under more realistic conditions the relationships calculated from the mathematical models, a mechanical model with elastic rather than infinitely compliant body walls was constructed. The "cloaca" was a toy balloon, connected by contact-cemented nylon monofilament "radial fibers" to a rubber condom "body wall" (Fig. 4). Cannulae to both "cloacal" and "coelomic" cavities were connected to pressure transducers on a Narco Biosystems polygraph, and to syringes for adjusting cloacal diameter and coelomic pressure. Cloacal pressure was recorded as a function of coelomic pressure at each of several cloacal diameters.

1



2

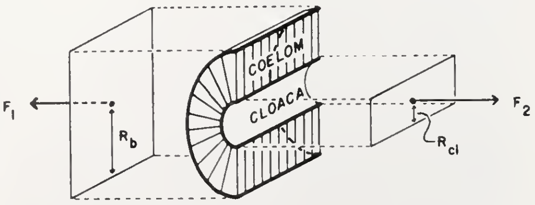
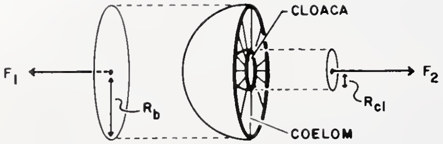


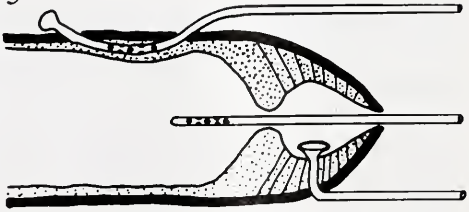
FIGURE 1. Cloacal pumps. Left: *Urechis caupo* (from life). Right: *Thyone briaereus*, a representative holothuroid (adapted from Hyman after Coe.)

FIGURE 2. Forces acting on a hydrostatically-supported body composed of coaxial cylinders.

3



5



4

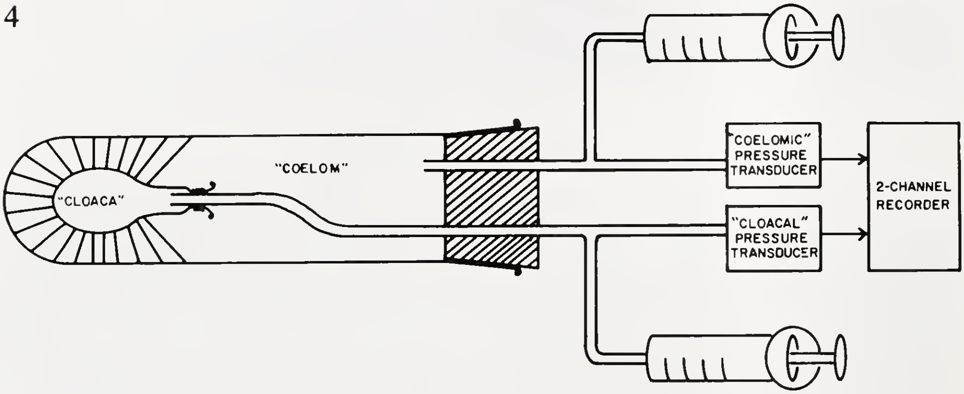


FIGURE 3. (Upper left) Forces acting on a hydrostatically-supported body composed of concentric spheres.

FIGURE 4. (Bottom) Rubber model of a cloacal pump.

FIGURE 5. (Upper right) Typical catheter placement for obtaining *in vivo* pressure records during cloacal pumping. Top: coelomic. Center: hindgut. Bottom: cloacal.

Experiments with living cloacal breathers were then undertaken to test the predictions based on the theoretical and mechanical models. Specimens of a common holothuroid (*Cucumaria miniata*) and a large (10–15 cm) echiuran (*Urechis caupo*) were obtained from the California coast. The animals were maintained in perforated 500 ml plastic containers in a 15°C recirculating sea water tank. Cannulae were fabricated of Intramedic 7410 polyethylene catheter tubing (Clay-Adams) by heat-forming. Cloacal cannulae were given a 90° bend and a large bell to retain them in the cloaca and bring the tube into line with the animal's axis (Fig. 5). Each coelomic cannula had a large ball melted onto its tip to anchor it, and transverse holes punched about 1 cm back from the tip with a sharpened piece of 25-gauge hypodermic tubing. Cannulae were inserted by thrusting an 18-gauge hypodermic needle through the body wall, threading the cannula through the lumen of the needle, and then withdrawing the needle, leaving the cannula in place. Successful implantation of cloacal cannulae and subsequent clearance from mucus blockage were verified by injecting carmine suspension through the cannula and observing its appearance in exhaled water; coelomic probes in *Urechis* were tested by aspiration of coelomic fluid and observation of pigmented coelomocytes.

Experimental animals were placed in 1-l dishes of 15°C sea water without restraint other than the cannulae. Coelomic and cloacal pressures were transduced to voltage with LX 1601D hybrid solid-state pressure transducers (National Semiconductor), conditioned by a variable gain/offset preamplifier, and recorded on a two-channel strip chart.

These procedures demonstrated once again that living animals are much less tractable research subjects than are mathematical models or rubber novelties, and great effort was required to collect useful data from a few successful preparations. The holothuroids consistently expelled their cloacal probes and successfully resisted emplacement of coelomic cannulae. The echiurans clogged their cannulae with mucus, ripped them through the fragile cloacal walls, and spent much effort attempting to rid themselves of the tubing while producing meters of irrelevant pressure records. The extremely thin respiratory hindgut of *Urechis* seemed to hinder accurate reading of coelomic pressures by wrapping around and occluding the holes in the cannula. Therefore, coelomic pressures were also recorded from cannulae threaded through the cloaca into the lumen of the hindgut (Fig. 5); this gave accurate readings but caused such abnormal behavior that little of the data was useful. Ultimately, only "free breathing" pressures were measured since occlusion of the anus triggered writhing, a "cough" reflex, and cessation of normal pumping.

RESULTS

In a cylindrical body plan of indefinite length (Fig. 2) the force F_1 perpendicular to the plane of section and tending to distend the body wall is the pressure differential across the body wall multiplied by the projected area of the body wall on the plane of section: $F_1 = P_{co}2R_bL$; where P_{co} is coelomic pressure referred by ambient, R_b is the body wall radius, and L is length. The force F_2 tending to collapse the cloaca toward the plane of section is the pressure differential across the cloacal wall multiplied by the projected area of the cloaca on the plane of section: $F_2 = (P_{co} - P_{cl})\ 2R_{cl}L$; where P_{cl} is cloacal pressure referred to ambient and R_{cl} is cloacal radius. At equilibrium these forces, coupled through the radial fibers, are equal in magnitude but opposite in sign (direction). Thus:

$$P_{co}2R_bL = (P_{co} - P_{cl})2R_{cl}L$$

Cancelling like terms, expanding the right-hand expression, and rearranging terms:

$$P_{cl}R_{cl} = P_{co}R_{cl} - P_{co}R_b$$

Solving for P_{cl}:

$$P_{cl} = P_{co}(1 - (R_b/R_{cl}))$$

In the spherical body plan (Fig. 3) the forces favoring distension are again balanced, equal to the product of pressure differential and projected area of the body wall and cloaca respectively. Thus:

$$P_{co}\pi R_b^2 = (P_{co} - P_{cl})\pi R_{cl}^2$$

Solving for P_{cl} as above:

$$P_{cl} = P_{co}[1 - (R_b/R_{cl})^2]$$

A similar solution would apply for a body plan between the two extremes; the exponent would be determined by the sphericity of the shape and would lie between 1 and 2.

Two relationships are apparent from the above analyses. First, suction attainable in the cloaca is strongly influenced by the relative size of cloaca and body wall, especially in more spherical bodies. As cloacal radius approaches that of the body wall, attainable suction approaches zero (P_{cl} approaches ambient). As cloacal radius decreases, attainable suction rises rapidly and, at cloacal radii smaller than one-half the body radius, exceeds the absolute value of coelomic pressure. The second, and perhaps more striking, relationship is that attainable cloacal suction is directly proportional to coelomic pressure. If coelomic pressure were released as Buddington (1937) suggested, inhalation would be impossible.

The results obtained with the rubber model (Fig. 6) are entirely consistent with the theoretical predictions. The cloacal suction is clearly proportional to coelomic pressure, and rises strikingly with decreasing cloacal filling (diameter). The suctions attainable at minimum cloacal diameters are remarkable—up to 20 times as large (absolute value) as coelomic pressure. These results again indicate that animals structurally similar to Figure 1 should not relax coelomic pressure during the inhalation stroke of their respiratory cycle. If anything, they might be expected to increase coelomic pressure, particularly if they are forced to suck in water against a pressure gradient. The maximum pressure against which they could suck in water should be related to the ratio of body wall diameter to cloacal diameter.

The pressure relationships in living animals during periods of apparently normal pumping confirm that no general relaxation of coelomic pressure occurs during the inhalation stroke of the respiratory cycle (Fig. 7). There is in fact no correlation between the cloacal and the coelomic pressure cycles, and inhalation may coincide with either a coelomic pressure peak or minimum. Cloacal pressures during inhalation were essentially equal to ambient, since the anus was unrestricted.

DISCUSSION

Both mathematical analyses and the mechanical model indicate that coelomic pressure must be maintained or even increased by contraction of the body wall musculature to produce cloacal suction. Why do *in vivo* recordings show no cor-

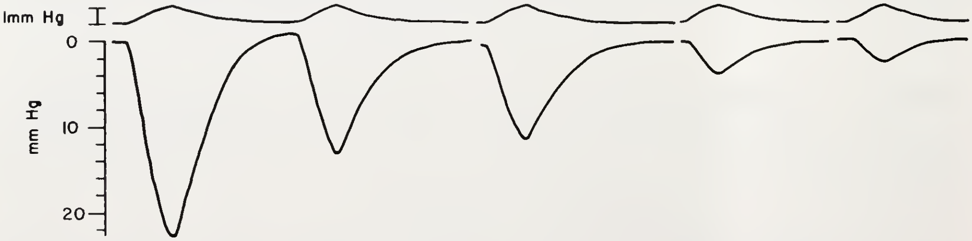


FIGURE 6. "Coelomic" (above) and "cloacal" (below) pressures in the model cloacal pump. Cloacal filling and therefore effective cloacal diameter increase from left to right. Coelomic pressure pulses 1 mm Hg, approx. 30s long.

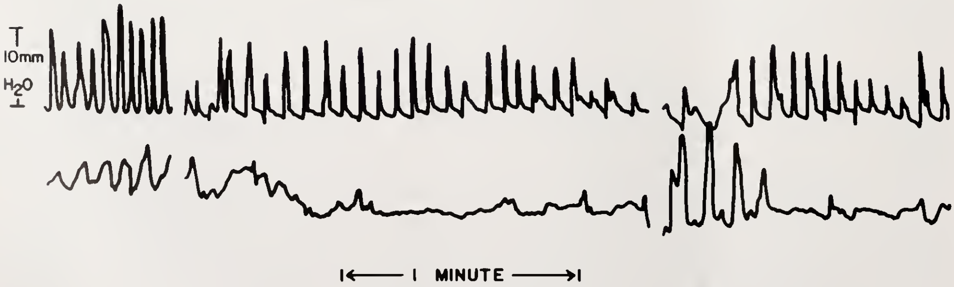


FIGURE 7. Cloacal (upper trace) and coelomic (lower trace) pressures in *Urechis caupo* during cloacal pumping.

relation between cloacal and coelomic pressures? The major reason is presumably that under normal conditions, and under the experimental conditions used, flow into the cloaca through the anus is unrestricted, and very low cloacal suction is required for filling. The increment in coelomic pressure required for radial muscle antagonism is minimal and apparently is masked by the changes in coelomic pressure associated with locomotory and burrow-ventilation movements; it is the latter that give rise to the pronounced rhythms in coelomic pressure in Figure 7. The fact that peaks in coelomic pressure may coincide with inhalation clearly demonstrate that relaxation of the body wall is not necessary for cloacal expansion. Indeed, such relaxation hinders pumping, as predicted. Some of the animals occasionally expelled all of the hindgut respiratory water and became completely flaccid. When they recommenced pumping, dimpling and collapse of the body wall at the points of radial muscle attachment were clearly evident, and cloacal filling was poor until enough hindgut volume had been accumulated to make the hydrostatic skeleton effective once again, confirming that adequate coelomic pressure is necessary for effective suction in *Urechis*.

This may not be the case in sea cucumbers, despite their architectural similarity to *Urechis*. It has long been known that sea cucumbers, while extremely limp when anesthethized or dead, are capable of rendering the connective tissue of the body wall quite rigid (Jordan, 1914, 1919). The facultative alteration of viscoelastic properties, an apparently unique property of echinoderm connective tissue, is caused by alteration of the ionic composition in the tissue matrix (Eylers, 1976; Wilkie, 1978) and gives the cucumber the ability to produce a semi-rigid exoskeleton, under nervous control and independent of coelomic pressure. Given the low suctions required for normal cloacal breathing, it is unnecessary to postulate a hydrostatic mechanism for supporting negative cloacal pressures in these animals, although such a mechanism may contribute to the process.

The mathematical analyses indicate that animals with a ratio of body wall diameter to cloacal diameter greater than 2:1 could theoretically produce cloacal suctions of greater magnitude than the coelomic pressure supporting the hydrostatic system. The mechanical model demonstrates that with appropriate body geometry a soft-bodied invertebrate could generate suction at least an order of magnitude greater than the coelomic pressure. The capabilities of the hydrostatic system appear to far exceed the minimal suctions required for cloacal pumping, and it is tempting to seek other animals in which the potential of such a system is more fully exploited. One group in which the ability to produce high suction would appear useful is the sand-eating deposit feeders like the hemichordate *Balanoglossus aurantiacus* or the polychaete lugworm *Arenicola marina*, both of which ingest remarkable quantities of sand. Wet sand is virtually impossible to push into a tube; however, suction will draw interstitial water into the tube, entraining an almost solid plug of sand. The requirements of these worms thus seem well matched to the capabilities of a hydrostatically-supported suction mechanism. Nevertheless, neither of them has the appropriate structure, and each uses a different technique for ingesting sand: ciliary action in enteropneusts (Barrington, 1940; Knight-Jones, 1953); proboscis eversion in *A. marina* (Jacobsen, 1967).

Nematodes are another group which relies on a hydrostatic skeleton and characteristically feeds by suction. In this case the structural similarities between the nematode buccal region and the *Urechis* cloacal region are somewhat more pronounced if the pharyngeal bulb is construed as a sac-within-a-sac separated by copious radial muscle, and it may be quite reasonable to invoke similar mechanisms for producing suction. Verification of such an hypothesis would present considerable difficulty, due to the small size of the cavities in which pressure would have to be monitored.

With the possible exception of the nematodes, then, no group of soft-bodied invertebrates seems to use a hydrostatically-supported mechanism for the production of high suction. It may be that development of these mechanisms has reached an evolutionary dead end—those animals that might find its capabilities advantageous do not possess the requisite morphology, while those which do have the structures have no need to fully exploit the potential high-suction capability of the system.

Although production of high suction requires a large ratio of body-wall radius to suction-cavity radius, a hydrostatically supported system also can produce low suctions with suction-cavity radii nearly as large as the body wall radius. In other words, if the pressure required is low, the stroke volume can be a substantial proportion of the total body volume. This property is exploited in the mantle of the squid, for jet propulsion as well as respiration. Large quantities of water are drawn in through wide inhalent openings and then expelled at high velocity through the narrow funnel; a complete cycle in *Loligo* may require less than 0.5 s (Ward, 1972). The mantle consists of an external and internal tunic, between which lie alternating bands of circular muscle fibers and radial muscle fibers connecting the two tunics (Ward and Wainwright, 1972). Water is expelled by rapid, synchronous contraction of the circular fibers. The mechanism by which the mantle is re-expanded has been the subject of some discussion; Ward (1972) dismisses positive-pressure pumping and Bernoulli effects, proposing that thinning of the mantle wall by contraction of the radial muscles must cause an increase in circumference, since the mantle does not lengthen and the volume of muscle tissue presumably does not decrease.

This mechanism for expansion of the squid mantle is a sac-within-a-sac system functionally identical with the cloacal pump of *Urechis*. The inner tunic corresponds to the cloaca, the outer tunic to the body wall, and the muscle layer (when the circular fibers are relaxed) behaves like a fluid within the observed range of deformation (20–30%; Ward, 1972). The suction potential of the mantle structure is slightly more than 10% of the hydrostatic pressure in the muscle layer (calculated by substituting measurements from Ward, 1972, in the above equation describing cylindrical animals). This potential suction is presumably well above what is required to draw in water through the unrestricted inhalent openings, although no pressure records from the mantle cavity or muscle layer are available. The hydrostatically-supported suction system permits the squid to produce only moderate suction, but more importantly it allows large stroke volume and rapid pumping through the mantle cavity without complex supporting structures that might interfere with streamlining, and without accessory pumps and valves that would introduce delays and additional fluid friction.

ACKNOWLEDGMENTS

My thanks to Dr. Steve Wainwright for encouraging me to develop these ideas. Partial support (instruments) came from NSF Grant DEB-77-16631 to the author. Contribution #80-4 from the Department of Marine Science and Engineering, N. C. State University.

LITERATURE CITED

BARRINGTON, E. J. W. 1940. Observations on feeding and digestion in *Glossobalanus minutus*. *Q. J. Microsc. Sci.* **82:** 227–260.

Bertolini, F. 1933. Sulle funzione dei polmoni acquatici delle Olothurie. *Pubbl. Staz. Zool. Napoli.* **13:** 1–11.

Buddington, R. A. 1937. The normal spontaneity of movement of the respiratory muscles of *Thyone briaereus. Physiol. Zool.* **10:** 141–155.

Crozier, W. J. 1916. The rhythmic pulsation of the cloaca of Holothurians. *J. Exp. Zool.* **20:** 297–356.

Eylers, J. P. 1976. The mechanical properties of Holothuroid body wall. *Thalassia Jugoslavica* **12:** 111–115.

Greenaway, P., and H. H. Taylor. 1976. Aerial gas exchange in Australian arid-zone crab, *Parathelphusa transversa* Von Martens. *Nature* **262:** 711–713.

Jacobsen, V. H. 1967. The feeding of the lugworm *Arenicola marina. Ophelia* **4:** 91–109.

Jordan, H. 1914. Über "reflexarme" Tiere. IV. Die Holothurien. 1 Mitteilung. Die Holothurien als hohlorganartige Tiere und die Tonusfunktion ihrer Muskulatur. *Zool. Jahrb.* Abt. 3, **34:** 365–436.

Jordan, H. 1919. Über "reflexarme" Tiere. IV. Die Holothurien. 2 Mitteilung. Die Reizbarkeit und die Einfluss des zentralen Nervensystems auf die Muskulatur und die muskelähnlichen Fasern der Haut (auf Erregbarkeit und Tonusfunktion). *Zool. Jahrb.* Abt. 3, **36:** 109–156.

Knight-Jones, E. W. 1953. Feeding in *Saccoglossus* (Enteopneusta). *Proc. Zool. Soc. Lond.* **123:** 637–654.

Newell, R. C., and W. A. M. Courtney. 1965. Respiratory movements in *Holothuria forskali* Delle Chiajei. *J. Exp. Biol.* **42:** 45–57.

Ogawa, S. 1928. A new approach used for the study of respiration in *Caudina chilensis. Sci. Rep. Tohoku Imp. Univ. (Sendai)* Ser. 4, **3:** 39–49.

Pantin, C. F. A., and P. Sawaya. 1953. Muscular action in *Holothuria grisea. Zoologia, Univ. S. Paulo* **18:** 51–59.

Parry, D. A. 1954. On the drinking of soil capillary water by spiders. *J. Exp. Biol.* **31:** 218–227.

Pearse, A. S. 1908. Observations on the behavior of the holothurian, *Thyone briaereus* (Leseur). *Biol. Bull.* **15:** 259–288.

Redfield, A. C., and M. Florkin. 1931. The respiratory function of the blood of *Urechis caupo. Biol. Bull.* **61:** 185–210.

Tao, Li. 1930. Notes on the ecology and physiology of *Caudina chilensis* (Muller) in Matsu Bay. *Proc. 4th Pacific Sci. Cong.* (1929) **3:** 7–11.

Ward, D. V. 1972. Locomotory function of the squid mantle. *J. Zool.* (*Lond.*) **167:** 487–499.

Ward, D. V., and S. A. Wainwright. 1972. Locomotory aspects of squid mantle structure. *J. Zool.* (*Lond.*) **167:** 437–449.

Wilkie, I. C. 1978. Nervously-mediated change in the mechanical properties of a brittlestar ligament. *Mar. Behav. Physiol.* **5:** 289–306.

Winterstein, H. 1909. Über die Atmung der Holothurien. *Arch. Fisiol.* **7:** 87–93.

Wolcott, T. G. 1976. Uptake of soil capillary water by ghost crabs. *Nature* **264:** 756–757.

Continued from Cover Two

high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

4. ***A condensed title*** or running head of no more than 35 letters and spaces should appear at the top of the title page.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text sould be headed LITERATURE CITED, and must be typed double spaced on separate pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin.* Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; the most recent issue). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** *The Biological Bulletin* has no page charges. However, authors will be requested to help pay printing charges of manuscripts that are unusually costly due to length or numbers of tables, figures, or formulae. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates or foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than correction of printers' or editors' errors).

CONTENTS

ALLEN, DENNIS M., AND WENDY B. ALLEN
Seasonal dynamics of a leech-mysid shrimp interaction in a temperate salt marsh 1
BELLON-HUMBERT, CHANTAL, FRANÇOIS VAN HERP, GODFRIED E.C.M. STROLENBERG, AND J. MANUEL DENUCE
Histological and physiological aspects of the medulla externa X organ, a neurosecretory cell group in the eyestalk of *Palaemon serratus* Pennant (Crustacea, Decapoda, Natantia) 11
CIMBERG, ROBERT L.
Variability in brooding activity in the stalked barnacle *Pollicipes polymerus* 31
CLARK, KERRY B., KATHE R. JENSEN, HUGH M. STIRTS, AND CESAR FERMIN
Chloroplast symbiosis in a non-elysiid mollusc, *Costasiella lilianae* Marcus (Hermaeidae: Ascoglossa (=Sacoglossa): effects of temperature, light intensity, and starvation on carbon fixation rate 43
DURLIAT, MICHELE, AND ROGER VRANCKX
Action of various anticoagulants on hemolymphs of lobsters and spiny lobsters 55
GOVIND, C. K., T. W. BUDD, AND H. L. ATWOOD
Fiber composition and innervation patterns of the limb closer muscle in the lobster *Homarus americanus* 69
HAYES, PAUL F., AND R. WINSTON MENZEL
The reproductive cycle of early setting *Crassostrea virginica* (Gmelin) in the northern Gulf of Mexico, and its implications for population recruitment 80
HERMAN, WILLIAM S.
Studies on the adult reproductive diapause of the monarch butterfly, *Danaus plexippus* 89
JARAMILLO, EDUARDO, JORGE NAVARRO, AND JÜRGEN WINTER
The association between *Mytilus chilensis* Hupe (Bivalvia, Mytilidae) and *Edotea magellanica* Cunningham (Isopoda, Valvifera) in Southern Chile 107
KUMAR, T. PAVAN, R. RAMAMURTHI, AND K. SASIRA BABU
Circadian fluctuations in total protein and carbohydrate content in the slug *Laevicaulis alte* (Ferussac, 1821) 114
NICKLAS, NANCY L., AND RICHARD J. HOFFMANN
Apomictic parthenogenesis in a hermaphroditic terrestrial slug, *Deroceras laeve* (Müller) 123
STOECKER, DIANE, R. R. L. GUILLARD, AND RHONDA M. KAVEE
Selective predation by *Favella ehrenbergii* (Tintinnia) on and among dinoflagellates 136
STUNKARD, HORACE W.
The life history, developmental stages, and taxonomic relations of the digenetic trematode *Lasiotocus minutus* (Manter, 1931) Thomas, 1959 146
STUNKARD, HORACE W.
The morphology, life history, and systematic relations of *Lasiotocus elongatus* (Manter, 1931) Thomas, 1959 (Trematoda: Digenea) 155
SUBRAMONIAM, T.
Protandric hermaphroditism in a mole crab, *Emerita asiatica* (Decapoda:Anomura) 161
THORINGTON, GLYNE, AND LYNN MARGULIS
Hydra viridis: transfer of metabolites between *Hydra* and symbiotic algae 175
WOLCOTT, THOMAS G.
Inhaling without ribs: The problem of suction in soft-bodied invertebrates 189

Volume 160 Number 2

THE BIOLOGICAL BULLETIN

Marine Biological Laboratory
LIBRARY
MAY 11 1981
Woods Hole, Mass.

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory
FREDERICK B. BANG, Johns Hopkins University
EDWARD M. BERGER, Dartmouth College
STEPHEN C. BROWN, State University of New York at Albany
HARLYN O. HALVORSON, Brandeis University
J. B. JENNINGS, University of Leeds
FRANK J. LONGO, University of Iowa
GEORGE O. MACKIE, University of Victoria
JOEL L. ROSENBAUM, Yale University
HOWARD A. SCHNEIDERMAN, Monsanto Company
DOROTHY M. SKINNER, Oak Ridge National Laboratory
J. RICHARD WHITTAKER, Wistar Institute of Anatomy and Biology
E. O. WILSON, Harvard University
GEORGE M. WOODWELL, Ecosystems Center, Marine Biological Laboratory

Managing Editor: CHARLES B. METZ, University of Miami

APRIL, 1981

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $10.00. Subscription per volume (three issues), $27.00 (this is $54.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor; or Susan Schwartz, assistant editor, at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and at the Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year.

Copyright © 1981, by the Marine Biological Laboratory
Second-class postage paid at Woods Hole, Mass., and additional mailing offices.
ISSN 0006-3185

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978). Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7⅝ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the

Continued on Cover Three

Reference: *Biol. Bull.*, **160:** 199–227. (April, 1981)

CIRCADIAN TIMING BY ENDOGENOUS OSCILLATORS IN THE NERVOUS SYSTEM: TOWARD CELLULAR MECHANISMS

JON W. JACKLET

Department of Biological Sciences and Neurobiology Research Center, State University of New York at Albany, Albany, NY 12222

CONTENTS

	Page
Abstract	199
Introduction	199
Entrainment and Coupling	200
Multioscillator Organization in Animals	202
Brain–Clock Connections	204
Gastropod Eyes	204
Arthropod Brains	207
The Pineal of Vertebrates	209
The Suprachiasmatic Nuclei of Vertebrates	211
Cellular and Molecular Mechanisms	212
Genetic Selection	212
Models	215
Macromolecular Synthesis, Proteins, and RNA	217
Literature Cited	223

ABSTRACT

The basic properties of circadian rhythms, such as oscillator type, entrainment to daily light-dark (LD) cycles, temperature compensation of the period length, and free-running periodicity, are remarkably similar in eucaryotic organisms from unicells to man. This encourages the view that all circadian oscillators are based on identical principles, found at the cellular level. Animals have a multioscillator organization, with brain centers (suprachiasmatic nuclei, optic lobes, etc.) and related structures (pineals, eyes, etc.) as sources of rhythmicity and coordination. Oscillators and driven activities are coupled by secretion (*e.g.* eclosion hormone, melatonin) or by direct neuronal connection. Oscillators of the multioscillator systems also are coupled. The cellular requirements for the circadian oscillator appear to be as generally uniform among various organisms as the basic properties. Ions and ion transport are important in the timing mechanism, as is protein synthesis on the eucaryotic ribosome. Although no concise model of the circadian oscillator encompassing protein synthesis, ions, and membranes has been offered, progress in analysis of the mechanisms has been made by genetic selection, screening of biochemical mutants, organ and tissue culturing, biochemical isolation of components, and chemical-pulse probing of the cellular oscillator.

INTRODUCTION

Anyone familiar with the cellular-level regulation of biological activities, *e.g.* insulin secretion, must be impressed with the excellent regulation of the complex

---

Non-standard abbreviations: CAP, compound action potential; CT, circadian time; DD, constant darkness; ERG, electroretinogram; LL, constant light; LD, light-dark cycle; NAT, N-acetyltransferase; PRC, phase response curve; SCN, suprachiasmatic nuclei.

processes involved. The more one knows about this regulation the more remarkable it seems. One thread (or more) of the web of regulatory processes in the cell is the endogenous circadian (about a day) clock. This clock controls overt cyclic activities in virtually all eucaryotes, from unicells to man. It is firmly established that circadian rhythms are a consequence of cellular oscillators. These oscillators are coupled to, and drive, such diverse activities as plant leaf movements, wheel-running in rodents, perch-hopping by birds, quantum catch of photoreceptors, and luminescent flashing of dinoflagellates. The precision of some of these overt rhythms reflects the precision of the underlying oscillator. For example, free-running locomotor activity in nocturnal rodents may show a standard deviation of 0.1 h (or 0.3%) out of a 23.9 h cycle period (Pittendrigh and Daan, 1976a).

Daily biological rhythms have been known for centuries. They were the subjects of enlightened enquiry by the astronomer De Mairan (1729) in the 18th century. They attracted the attention of eminent biologists such as Charles Darwin (1881). But only comparatively recently has the term circadian (coined by Halberg, 1960) come into common use in reference to a specifically defined phenomenon (Aschoff, 1965), and a vigorous search for the cellular basis of circadian rhythms begun. Approaches to discovering the mechanisms were considered in detail at the Dahlem Conference (Hastings and Schweiger, 1976). The present paper reviews the state of the search for circadian clock sites in animals, and for cellular and molecular bases of the circadian oscillator. This cellular emphasis complements other reviews (Block and Page, 1978; Rusak and Zucker, 1979) of the nervous system's participation in circadian rhythms.

Daily changes in environmental lighting and temperature result from the earth's rotation around its axis, and seasonal changes in these variables result from the earth's tilt as it orbits the sun. These fluctuating environmental stimuli strongly influence activities of plants and animals, resulting in driven activities that change with exactly the periodicity of the earth's rotation (24 h). In the absence of solar-day cues (*e.g.* in constant darkness and temperature) many of these activities continue to be periodic, but the cycles are only *about a day* (circa-dian). Periods of 21–26 h are common. This persistent cycling is the primary defining characteristic of circadian rhythms. Rhythms that require periodic stimuli, that do not continue in constant conditions, and that therefore lack an endogenous oscillator, are by definition not bona fide circadian rhythms. A complete definition (Hastings *et al.*, 1976), includes, among other things, compensation for temperature in the period length, and entrainment of the rhythms by environmental time-givers, such as light and temperature.

Since the characteristics of rhythms in unicells and man are nearly identical, circadian rhythms are thought to have been conserved during evolution, and to be of adaptive consequence. The biological usefulness of rhythms may be appreciated by considering the four categories of biological time measured by circadian clocks (listed by Pittendrigh, 1976): (1) programming a daily sequence of metabolic and behavioral changes, (2) enabling an animal to recognize a specific time of day and to return at that time on subsequent days, (3) enabling maintenance of a constant compass heading using the sun's azimuth as reference, and (4) making it possible to distinguish different durations of light and darkness as a measure of season (photoperiodism).

Entrainment and coupling

The circadian timer has an inherent period of about a day. It is brought into exact conformity with the solar day's environmental changes by the process of entrainment, which advances or delays the phase of the endogenous oscillator at times in its cycle when it is sensitive to an environmental time-giving stimulus. The primary time-giver stimulus is light, although other stimuli also are effective in some systems. Typically, circadian clocks are most sensitive to light during the subjective night, the time in the circadian cycle when night would occur if the organism were exposed to a LD cycle. During early subjective night, light delays the phase of later cycles. During late subjective night, light advances the phase of latter cycles. The result is stable entrainment of the oscillator to a light-dark (LD) cycle, such as the solar LD cycle (Pittendrigh, 1974). The circadian oscillator's responses to light pulses at different phases of the cycle can be plotted against phase in the cycle to obtain a phase response curve (PRC). A PRC describes the underlying oscillator (Pittendrigh, 1974), since it characterizes the succession of points in the cycle in terms of sensitivity to the time-giver. As will be discussed below, the PRC obtained by responses to chemical agents is also a useful way to describe the effects of those agents on the timing mechanism.

Measuring the locomotor activity of a hamster in a running-wheel convinces one of the precision and reliability of circadian rhythms. But it leaves in question the driving oscillator's location and the mechanism that couples it to overt activities. Some answers have been gained by various techniques; creating lesions in specific tissue, isolating tissue within the organism, removing and then replacing tissue, and isolating tissue outside the organism, in organ or cell culture.

Many unicells have special advantages for particular analytical approaches. Therefore, the organization of rhythms in two such organisms, *Gonyaulax* and *Acetabularia*, will be examined before considering the complexity of metazoan rhythms.

The dinoflagellate *Gonyaulax*, has rhythms in photosynthetic capacity, cell division, glow, and luminescence, among others (Fig. 1A). These rhythms have different phase relationships in the circadian cycle. But all appear driven by the same basic oscillator, because they maintain a constant phase relationship and periodicity in free-running conditions, and change in phase to the same extent after a time-giver stimulus (McMurray and Hastings, 1972). Krasnow *et al.* (1980) recently examined their spontaneous light-emitting rhythm. It consists of a glow rhythm and a flashing rhythm. The phase difference between these two changes with time in constant conditions and depends upon light intensity. This raises the possibility of control by more than one oscillator.

The giant single-cell alga, *Acetabularia*, has several measurable rhythms, including O_2 production, chloroplast migration, and extra-cellular electrical currents. With its nucleus removed, the cell will survive and still have good rhythms. Since rhythms in unicells are usually measured on populations in culture, it has been asked if each cell is capable of expressing a rhythm in isolation, and to what extent individuals in a population interact in generating a rhythm. Individual cells of *Acetabularia* (Karakashian and Schweiger, 1976a) or *Gonyaulax* (Sweeney, 1960) apparently express a circadian rhythm. Individuals of a population interact very little, judging by the spread in the distribution of period lengths on successive days of free-running activity.

The *Gonyaulax* rhythms shown in Figure 1 illustrate the general concept that several activities may be coupled to the basic cellular oscillator. The observable rhythmic activities are quite separate from the basic oscillator, but they serve as indicators (or "hands") of the clock's performance. Selectively suppressing one of these activities does not perturb the clock (Hastings, 1960). This suggests that the

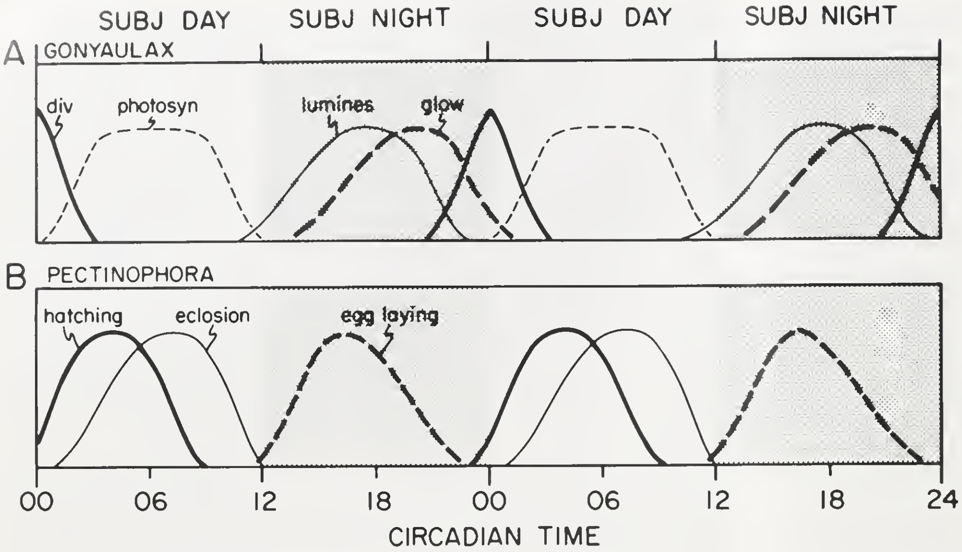


FIGURE 1. Overt rhythmic activities coupled to the circadian clock. In A, the four separate activities of cell division (div.), photosynthetic capacity (photosyn.), bioluminescence (lumines) and glow exhibited by a *Gonyaulax* culture are shown. All four are coupled to the same cellular clock, since they maintain a fixed phase relationship. B shows three separate activities, egg hatching (hatching), egg laying, and pupal eclosion, exhibited by the moth *Pectinophora* at separate stages in the life cycle. The curves in B are the distribution of events for a population of organisms. Two days of circadian time in hours are shown, divided into subjective day and subjective night. Redrawn from McMurray and Hastings (1972) and Pittendrigh (1976).

activities themselves do not influence the mechanism by feedback and are, therefore, not part of the circadian timing device.

Activities that occur only once in the organism's lifetime, as well as ongoing daily activity rhythms, are coupled to the basic oscillator. An example among metazoans is the moth *Pectinophera*, where the circadian clock times egg laying, egg hatching, and pupal eclosion, which occur at different stages of the life cycle and different phases of the circadian cycle (Pittindrigh, 1976; Fig. 1B). Members of a moth population will perform these activities with a temporal distribution like that shown in the figure. For example, some moths will go through eclosion on one day at a certain time of day. Other moths may do it on the next day, but always at the appropriate time.

Multioscillator organization in metazoa

If each unicellular organism has a circadian oscillator, each cell in a multicellular organism may have at least the potential for it. But instead of each metazoan cell going its separate way, specific sites, arranged hierarchically, appear to be important in generating rhythms. Intact animals kept under constant conditions, so that circadian rhythms are free-running, reveal both the multioscillator nature of the circadian organization, and interactions between oscillators. In man, activity-rest cycles proceed with the same periodicity as other rhythms of ion excretion, body temperature, etc. However, after some days without time cues, these rhythms may break away from one another (Fig. 2) and continue at their own periodicity

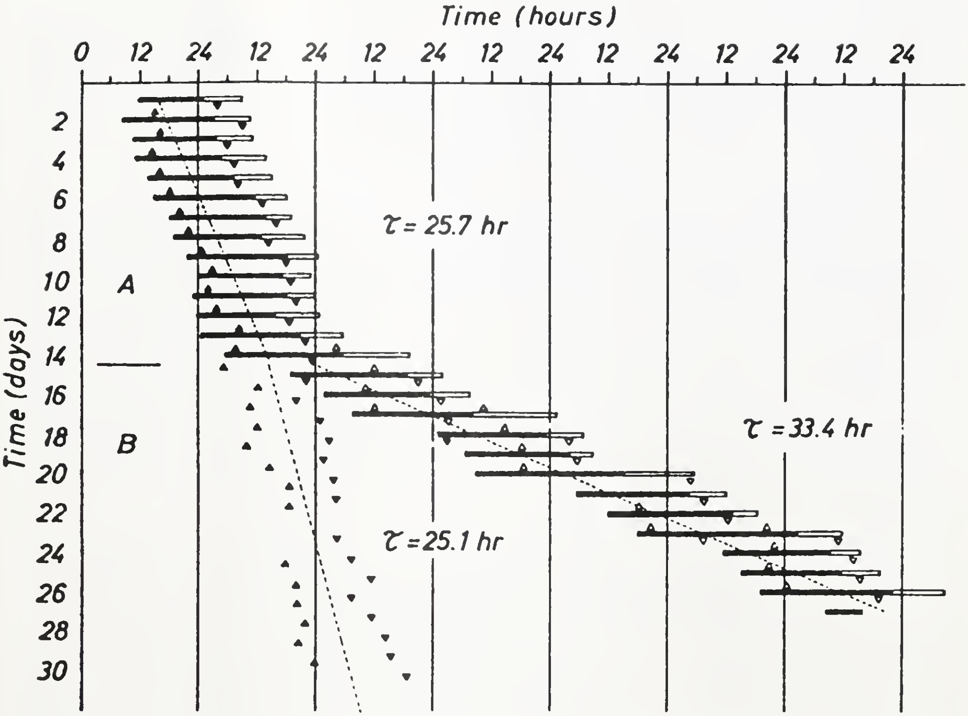


FIGURE 2. Rhythms of a human subject under constant conditions without time cues. Successive days of activity are plotted downward against a scale of solar time. The bars are activity (black) and rest (white). The triangles are maxima and minima of rectal temperature. The free-running periods (τ) are 25.7 h before the spontaneous desynchronization (A) and 33.4 h for activity and 25.1 h for temperature afterwards (B). From Wever (1975).

(Aschoff and Wever, 1976; Wever, 1979). This internal desynchronization shows the existence of several circadian pacemakers usually coupled to one another. Figure 2 shows an example in which activity and rectal temperature were proceeding at the same periodicity, 25.7 h. After desynchronization, each rhythm continued with a different period (33.4 h and 25.1 h) than the previous 25.7 h period, suggesting that the clocks were interacting.

Another phenomenon observed in mammals kept under prolonged constant conditions is "splitting." The activity-rest cycle proceeds with a normal free-running periodicity for some time and then abruptly splits into two activity bands instead of one, the two adopting a 180° anti-phase relationship. This again is evidence for more than one pacemaker. To account for "splitting" and other circadian phenomena, Pittendrigh and Daan (1976b) proposed a circadian system having two kinds of oscillators, with opposite dependencies.

Something similar to "splitting" has been observed in the rhythms of neuronal activity in *Aplysia* eyes. Each eye has the complete organization of a circadian clock. If intact animals are entrained to light-dark cycles, the two circadian clocks adopt identical phases. However, if the animal is placed in constant dim light for days, the eyes adopt different phases (Hudson and Lickey, 1980). The tendency toward 180° anti-phase relationship similar to the "splitting" observed in mammals suggests weak coupling between the separate clock sites.

THE BRAIN–CLOCK CONNECTION

Discrete tissues devoted to the clock function profoundly affect overt circadian activities. In animals, these tissues are brain centers or related structures commonly associated with light reception and neurosecretory activity.

Gastropod eyes

The "sea hare," *Aplysia*, is an example of gastropod circadian organization whose cellular and hierarchical organization has been studied in considerable detail. *Aplysia* became a recognized model for circadian studies with the pioneering work of Strumwasser (1965) on a single identifiable neuron. Intracellular recordings of membrane electrical activity seemed to show a bona fide circadian rhythm, but later tests showed that the neuron failed to sustain a circadian rhythm (Beiswanger and Jacklet, 1975; Lickey *et al.*, 1976). This discouraged the use of that single neuron as a suitable experimental preparations, and leaves in doubt the existence of any circadian rhythm in it. A single neuron exhibiting a distinct circadian rhythm has yet to be found, but the idea of a cellular clock coupled to and driving membrane electrical activity was established.

Further study revealed a robust circadian oscillator system in the *Aplysia* eye (Jacklet, 1969a). Rhythm in electrical activity was recorded from the isolated eye-optic nerve. This established that the rhythm is generated by a discrete organ, and raised the possibility of testing the animal's circadian abilities in the presence and absence of this known clock. The eye of *Aplysia* is small and inconspicious, as perhaps befits an animal with limited visually oriented behavior. The eye, a closed-vesicle type, has a central lens surrounded by a complex retina of several thousand neurons and photoreceptors. The long optic nerve is convenient for electrical recordings, made continuously while the eye is maintained in controlled environmental conditions in an organ culture. The most conspicious activity recorded from the optic nerve is compound action potentials (CAP) which represent the synchronous firing of a population of neurons (Jacklet, 1969b, 1973). CAPs are evoked by light or occur spontaneously in darkness. Dark activity changes in a rhythmic way, as shown in Figure 3, and continues to do so for 2 weeks or more in isolation. The period of the rhythm depends upon the composition of the culture medium: In artificial seawater alone the period is 23–24 h. In a nutrient medium the period increases to 26–28 h (Jacklet, 1971), depending primarily upon amino acids present in the medium.

Figure 3 shows the circadian rhythm as recorded in constant darkness at 16°C. The time axis is in circadian time (CT), which refers to 24 circadian h for each cycle of the rhythm. In this case the period length is 26 h of solar time, so each circadian hour is equal to slightly more than one solar day hour. In circadian time (CT) the 00 hour refers to subjective dawn, the time when dawn would have occurred in the circadian cycle. Dusk is then 12 h, and 18 h is the middle of the subjective night. Using this notation, one can appreciate that electrical CAP activity normally begins before dawn (anticipation). This activity remains high during the subjective day and becomes low during the subjective night.

Endogenous CAP activity in complete darkness, sampled at several circadian times, is shown in Figure 3B. As activity commences at CT 22, CAPs occur at regular but long intervals. At CT 00, when the frequency is half its maximum rate, CAPs occur at shorter intervals and are clustered in "bursts" of two or three. At CT 02, the frequency is near maximum, activity is clustered into bursts of four, and the amplitude of the CAPs is more than twice that at CT 22. Activity is still high at CT 05 but diminished at CT 21. The activity subsides by decreases in the

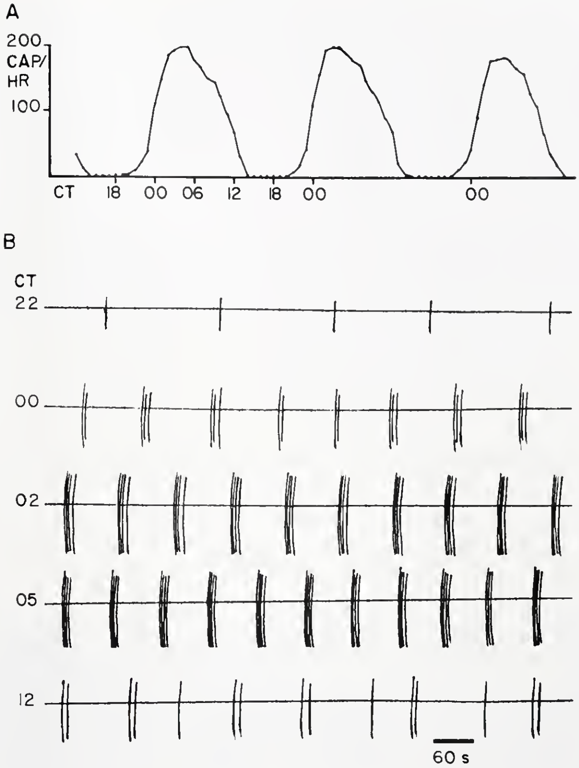


FIGURE 3. Circadian rhythm of endogenous compound action potential (CAP) frequency recorded from the optic nerve of an isolated *Aplysia* eye in organ culture during constant darkness. In A, the frequency is plotted against circadian time (CT). B shows the actual CAP record representative of each CT (22, 00, etc.). Note the changes in amplitude as well as frequency with advancing CT. The firing mode also changes, from pacemaking to "bursting." Largest CAPs are 100 μV and time scale is 60 s in B. Jacklet, unpublished.

number of CAPs per burst and later by increases in the interval between bursts, similar but in opposite sequence to the increases.

Intracellular circadian oscillators probably control the observed CAP firing, since the membrane CAP activity can be artificially suppressed or enhanced without changing the period or phase of the circadian rhythm (Jacklet, 1973; Eskin, 1977). The CAP pacemaker or "bursting" activity, which occurs at intervals of minutes, is produced by a separate membrane oscillator involving changes in ionic conductance. The circadian oscillator modulates the CAP "bursting" activity in a systematic way. One imagines the membrane conductances and/or ion transport properties of each CAP-generating neuron being altered over the circadian cycle by couplings to the intracellular circadian oscillator, with silence impressed at one phase and bursting pacemaker activity induced at the other.

In *Aplysia*, the active portion of the activity cycle corresponds to the active phase of locomotor activity. Simultaneous recordings of optic nerve activity and locomotor activity from intact, freely moving sea hares show that locomotor onset closely follows the CAP activity that anticipates dawn (Block, 1979). The eyes are not the critical photoreceptor for the diurnal locomotor activity of *Aplysia*, since eyeless animals remain diurnal on light-dark cycles at 200 lux, but do not anticipate dawn (Lickey *et al.*, 1976). However, the eyes are an important influence when locomotor activity is allowed to free-run in the absence of a forcing LD cycle. Periodicity deteriorates in eyeless animals (Strumwasser, 1973), although some still have weak periodicity (Lickey *et al.*, 1977). It appears that the eyes are the major pacemakers for circadian locomotor activity, but that other weaker sources also influence this activity (Lickey and Wozniak, 1979; Strumwasser *et al.*, 1979).

Intact neural connections from the eye to the central nervous system are necessary in order for the eye to influence locomotor activity (Lickey *et al.*, 1976). This is true even though the eye secretes polypeptides (Harf *et al.*, 1976) and some of the peptides (~1000 MW) are released rhythmically in phase with the rhythm of CAP activity (Strumwasser *et al.*, 1979). The target of this secretion is not known.

The synchronous firing of retinal neurons (CAP activity) may efficiently release neurosecretory material (Jacklet, 1969b). This idea is supported by the finding that CAPs are produced by the secondary or D (dark) neurons, shown in Figure 4 and identified by dye injection and intracellular recording (Jacklet, 1976, 1979). These neurons have the ultrastructural characteristics of neurosecretory cells, including dense core vesicles (Luborsky-Moore and Jacklet, 1977; Strumwasser *et al.*, 1979).

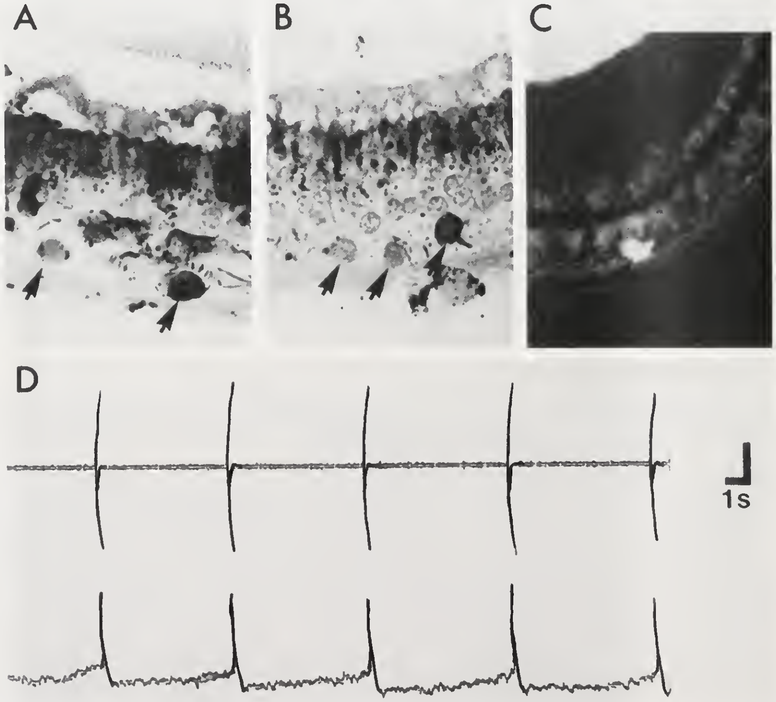


FIGURE 4. Output neurons of the circadian oscillator in the *Aplysia* eye. A, B, and C are histological sections of the retina showing receptors in the pigmented layer and secondary or D neurons (arrows) below. Some of the neurons (~15 μm in diameter) are backfilled with cobalt in A and B. The neuron in C was filled with Lucifer yellow, a fluorescent dye, by intracellular injection. Only the D neuron is filled with dye; other light areas of the retina are autofluorescence. In D, the endogenous activity from the optic nerve (top) and a simultaneously impaled "D" neuron (bottom) is shown. Time scale is 1 s and voltage is 20 μV, top, and 10 mV, bottom. From Jacklet and Schuster, unpublished.

Rhythmic CAP activity probably is responsible for the rhythmic polypeptide release shown by Strumwasser *et al.* (1979). Peptides or catechol amines (Luborsky-Moore and Jacklet, 1977) may be released at the terminals of the optic nerve in the cerebral ganglion, since ligation of the optic nerve leads to accumulation of fluorescent material on the eye side of the ligation. The eye contains serotonin and lesser amounts of dopamine. Pulses of exogenous serotonin phase shift the eye rhythm (Corrent *et al.*, 1978).

The circadian clock organization observed in *Aplysia* eyes is not characteristic of all gastropod eyes, but other examples are known. The eye of *Navanax* is similar to *Aplysia's* (Eskin and Harcombe, 1977): the optic nerve CAPs are driven in an endogenous rhythm. Comparable organization holds for the eye of *Bursatella*, the frilled sea hare, where the period of the rhythm is somewhat shorter (21 h compared to 24 h in *Aplysia*) and the effect on locomotion is less distinct (Block and Roberts, 1980).

Arthropod brains

The optic lobes of the cockroach are the site of the circadian clock controlling its locomotor activity, and its compound eye is the photoreceptor that mediates light entrainment of the oscillator (Brady, 1969). Lesions of the optic lobe implicate the lobula as the site (Roberts, 1974; Sokolove, 1975). An intact neural pathway from the optic lobes to the thoracic ganglia is necessary for expression of the rhythm. Therefore, the coupling is believed to be neural. In addition, attempts to repeat earlier studies showing humoral coupling failed (see Brady, 1969).

In crickets, three rhythms have been studied: locomotion, stridulation, and spermatophore production. The clock appears to be in the optic lobe. It is entrained via the compound eyes, and neural connections from the optic lobes to the brain are necessary. The pars intercerebralis serves as a coupling site between the optic lobe oscillator and the various behaviors. The channels from the pars intercerebralis may be neural or humoral (Sokolove and Loher, 1975).

Among insects, as among gastropods, clock sites and coupling mechanisms are not identical. In moths, the flight activity rhythm is controlled by a cerebral lobe clock, not the optic lobe, and entrained via extraocular receptors in the brain itself. It is neurally linked to the thoracic motor centers for flight (Truman, 1974). Contrasting with this arrangement is the moths' control of eclosion behavior. Again the clock is in the cerebral lobes and a brain receptor mediates entrainment by light. However, the coupling between timer and eclosion is via a humoral agent, the eclosion hormone. This arrangement was demonstrated by transplanting the brain from one animal into the abdomen of a debrained animal (Truman, 1972) and observing that eclosion occurred at times appropriate for the transplanted "loose brain" in the abdomen, and that the circadian phase of subsequent eclosion behavior was appropriate for the brain's clock. Thus, transplanting the brain clock transferred the phase. The eclosion hormone triggers programmed neuronal activity from the abdominal nervous system, which directs eclosion behavior (Truman, 1979).

Handler and Konopka (1979) recently demonstrated another case of humoral coupling, in *Drosophila.* Transplanting a brain from a clock-period mutant fly caused an arrhythmic host fly receiving the transplant to assume the periodicity expected of the transplanted mutant brain. Here, a humoral coupling, rather than the direct neural connection presumed for most other insects studies, apparently drives the circadian locomotor activity.

The eye of *Limulus*, the horseshoe crab, has been studied extensively from the standpoint of visual physiology and feature extraction. But only recently has it been recognized (Barlow *et al.*, 1977) that a circadian clock affects the eye's performance. Both electroretinogram (ERG) and optic nerve responses of the lateral eye change in magnitude in response to standard light pulses. Highest firing rates of optic nerve fibers are obtained at night, when efferent nerve activity from the brain to the eye is high (Fig. 5). The animal moves most at night. Normally, a circadian clock in the brain controls the rhythmic change in efferent activity, but selectively shocking the optic nerve with electrical pulses activates the efferent fibers and elevates the amplitude of the visual response. It appears, therefore, that the efferent fibers couple the clock output to the light receptors. This effect has been demonstrated in single retinula cells (Kaplan and Barlow, 1980). The efferent activity decreases the photoreceptor noise (quantum bumps) and increases the photoreceptor response (receptor potential) to light. The response increases partly because changes in the morphology of the retinula and surrounding pigment cells maximally expose the photosensitive rhobdom to light. But the mechanism for reducing noise is obscure.

The efferent activity controlled by the circadian oscillator also is responsible for enhanced turnover of the photosensitive membranes. Onset of first light, at dawn, causes synchronous breakdown and then reassembly of these membranes, but this process can be blocked by blocking the efferent optic nerve activity (Chamberlain and Barlow, 1979). The morphology of the ommatidial cells also changes under the influence of the circadian clock in the brain (Barlow *et al.*, 1980).

Crustaceans also show rhythmic changes in light responsiveness and locomotor activity. Aréchiga and Wiersma (1969) reported an interesting circadian rhythm (period 22–24 h) of electroretinogram amplitude and activity of single visual units (sustaining fibers) in crayfish. Sensitivities are highest during the nocturnal peaks in locomotor activities. The various rhythms observed are in phase and appear to

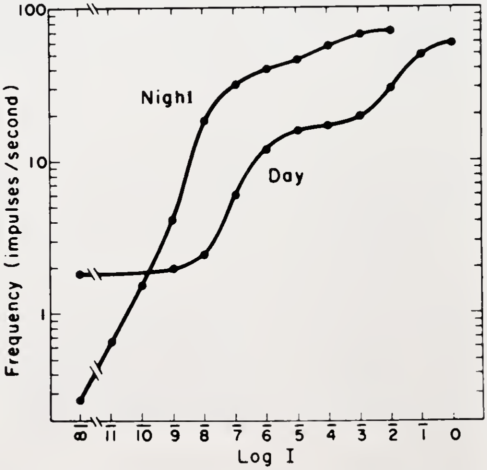


FIGURE 5. Differences in firing rates of a single optic nerve fiber of *Limulus* eye in response to standard test flashes. The animal was in continuous darkness between flashes. "Day" responses were recorded from 1500–1600 h and "night" responses from 2100–2200 h. Modulation by the circadian clock, carried by efferent fibers to the eye, is responsible for the shift in sensitivity at "night." Log I = 0 is 10^{12} quanta/s at cornea incident on the single ommatidium from 400–650 nm. From Barlow *et al.*, 1977, copyright 1977 by the American Association for the Advancement of Science.

be driven by a common clock mechanism and mediated by a humoral substance. Support for humoral mediation comes from experiments showing that injecting eyestalk extracts reproduces daytime phase characteristics (quietness) (Aréchiga *et al.*, 1974). The eyestalk factor, also found elsewhere in the nervous system, is proteinaceous and depresses neuronal activity—hence its name, neuro-depressing hormone. A variety of crustaceans may use the same substance, since there is cross-sensitivity among species (Aréchiga *et al.*, 1979).

The pineal of vertebrates

Pineal organs, which arise from evaginations of the diencephalon of the brain, are one class of circumventricular organs. Structure and innervation of pineals differ pronouncedly in lower and higher vertebrates (Wurtman *et al.*, 1968).

The pineal gland of the sparrow contains a circadian oscillator. When kept in constant darkness, sparrows deprived of their pineal glands become arrhythmic, and lack the periodic circadian locomotor activity of normal birds. However, pinealectomized sparrows appear to entrain to light-dark cycles rather than being directly driven by the cycles, since such birds anticipate lights-on. This suggests that other "damped" oscillators may directly drive locomotion. An alternative is that other oscillators (*e.g.*, the suprachiasmatic nuclei) depend upon the pineal for coupling their action to locomotion (Takahashi and Menaker, 1979). To know a sparrow is not to know all birds, however. Pinealectomy disrupts the system but does not cause permanent arrhythmia in starlings (Gwinner, 1978), and gallinaceous birds are not affected.

When Zimmerman and Menaker (1979) transplanted pineal glands into the anterior chamber of the eye of pineal-less birds, the recipient birds regained a circadian rhythm. Furthermore, the phase angle of the rhythm, measured from the onset of activity in the host animal after the transplant, was as expected if the transplanted pineal contained the clock. Thus, the transplantation did not significantly perturb the clock in the pineal, and the recipient bird's locomotor activity quickly became coupled to it, presumably by the pineal secretion melatonin.

The pineal of the chicken is similar: When it is isolated in organ culture, the circadian rhythm of melatonin and associated enzymes of the biochemical pathway persist (Binkley *et al.*, 1978). Melatonin is synthesized in the pineal by a well known biochemical pathway (Klein, 1974). The amino acid tryptophan circulating in the blood is converted to 5-hydroxytrytamine (serotonin) in the pineal. Serotonin is converted by the enzyme N-acetyltransferase (NAT) to N-acetylserotonin, which is acted on by hydroxyindole-o-methyl transferase (HIOMT) to produce melatonin. Melatonin is produced rhythmically by this pathway, with production highest during the dark and lowest during light. The two enzymes, HIOMT and especially NAT, also change in activity rhythmically. The strong rhythm in NAT activity seems largely responsible for the rhythmic changes in serotonin and melatonin concentrations.

Isolated perfused chicken pineal glands *in vitro* release melatonin rhythmically (Takahashi *et al.*, 1980). When individual pineal glands of 5–8-week-old chickens were placed in constant darkness, the rhythm continued, but with a reduced amplitude (Fig. 6). These experiments show the pineal has an endogenous circadian oscillator that may be coupled to other activities by melatonin secretion.

Deguchi (1979) maintained dissociated chicken-pineal tissue in a cell culture. He found that NAT activity from the dissociated pinealocytes was rhythmic: activity was highest in darkness and lowest in light. NAT activity persisted as an

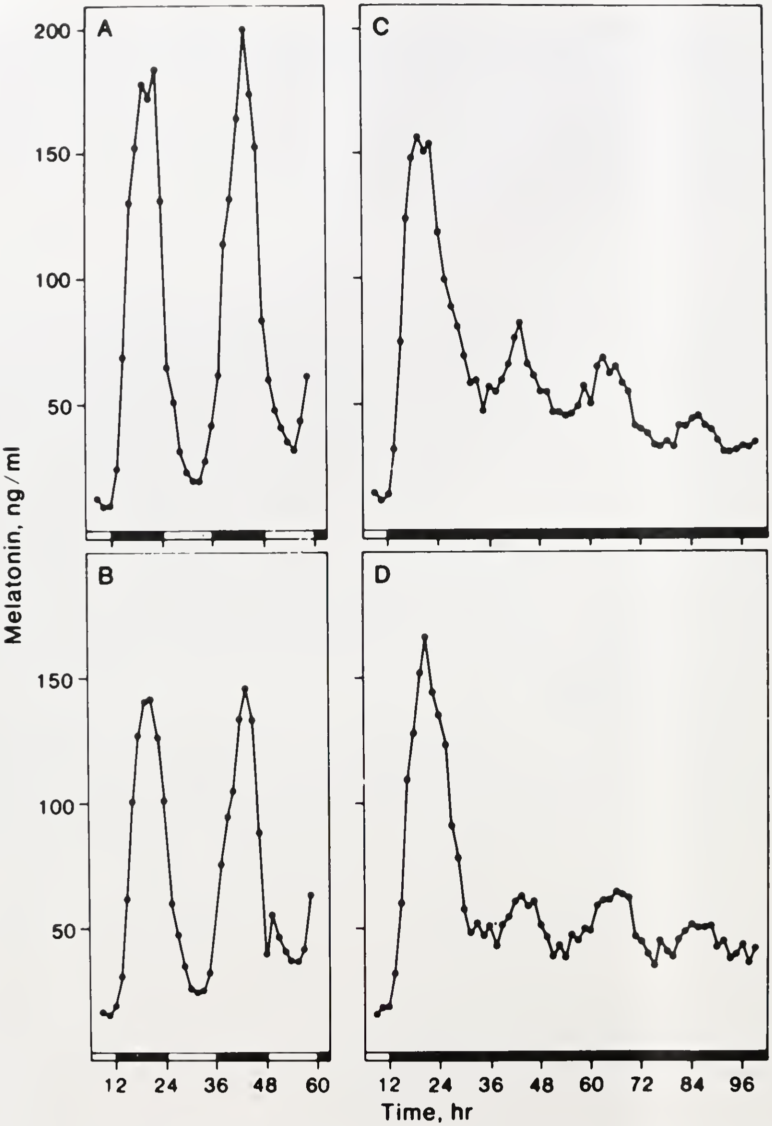


FIGURE 6. Rhythms of melatonin release from isolated perfused chicken pineals. A and B are in light-dark cycles and C and D are in constant dim red light (1–5 lux). Each record is from one pineal. In the constant dim light the rhythms persist, although damped, showing the presence of an endogenously timed release mechanism. From Takahaski *et al.*, 1980.

endogenous circadian rhythm in constant darkness. It entrained to a reversed photocycle. This establishes that the cell culture contains both a circadian clock and the photoreceptor that entrains the clock to light. Since the dissociated pinealocytes are not in an organized tissue, these results also suggest that each cell has its own photoreceptor and circadian oscillator, coupled to the enzyme NAT. Romero *et al.* (1975) blocked the rat pineal's rise in NAT activity in darkness with cycloheximide, showing protein synthesis is necessary. RNA synthesis is also required at some times in the cycle.

The rat pineal also produces melatonin rhythmically, but the circadian oscillator is not in the pineal. Oscillators in the suprachiasmatic nuclei drive the pineal rhythm (Moore and Klein, 1974). The two are coupled via neural connections of the sympathetic nervous system. The neurotransmitter of the sympathetic system, norepinephrine, is released and combines with β-receptors on the pineal. This activation of the β-receptors causes c-AMP production, which promotes NAT synthesis. This forced rhythm of the rat pineal, driven by the suprachiasmatic nuclei (SCN), contrasts with the endogenous oscillator in the chicken pineal.

Melatonin is also synthesized in the retinas of many vertebrates. Recently, circadian rhythms of melatonin content and NAT activity have been shown in chick retina (Hamm and Menaker, 1980). These rhythms persist in constant darkness and are not abolished by pinealectomy. The levels of NAT and melatonin in the retina are similar to those found in the pineal. Light inactivates NAT activity, as it does in the avian pineal.

Melatonin rhythms may promote or modulate rhythms of shedding of outer segment disks from retinal rods. Melatonin rhythms also could affect pigment migration, and photomechanical movements of rods and cones.

The suprachiasmatic nuclei of vertebrates

Ablation identified the SCN of the mammalian brain as an important circadian oscillator (Moore and Eichler, 1971; Stephen and Zucker, 1972). These bilaterally symmetrical nuclei of the hypothalmus lie just above the optic chiasm (Fig. 7) and receive light information by a retinohypothalmic tract. They control mammalian rhythms such as locomotion and pineal secretion, and are near the top of a hierarchy of oscillators that control mammalian rhythms and integrate the activity of other suspected oscillators into a circadian framework (Rusak, 1979).

An improvement over SCN ablation uses a Halasz knife to surgically isolate, but not remove, an island of rat hypothalmic tissue containing the SCN (Inouye and Kawamura, 1979). Recordings inside the island and at neighboring hypothalmic sites showed that circadian neuronal activity persisted only inside the island.

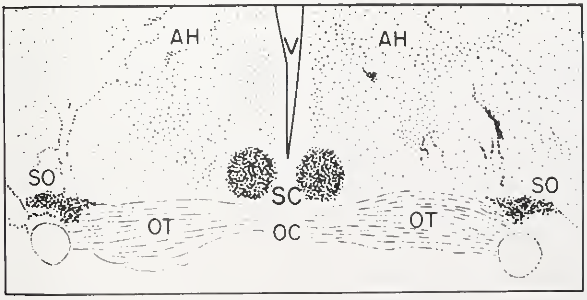


FIGURE 7. Suprachiasmatic nuclei of the rat are shown in a frontal section through the hypothalmus. The nuclei have been selectively ablated, isolated as an island of tissue, and studied by the deoxy-glucose method. These studies implicate them as circadian clock sites. Suprachiasmatic (SC) and supraoptic (SO) nuclei, optic chiasm (OC), optic tract (OT), anterior hypothalmus (AH) and third ventricle (V) are shown. Adapted from Moore and Klein, 1974; Inouye and Kawamura, 1979.

More distant sites, such as the raphe, substantia nigra, and reticular formation, also lost their rhythmicity. Normally, these rhythmically active brain structures are most active during projected night, when rats are most active. Some recordings from the hypothalmic island showed maxima at night but others had maxima at day. Later, histological examination of the brain recording sites showed that recordings with day maxima were from the SCN proper, while those with night maxima were from other sites within the island. The rhythmic activity in tissue outside the SCN is 180° inverted in phase compared to the SCN activity. The maximum SCN activity during projected day corresponds to the maximum 2-deoxy-D-glucose uptake in the SCN during projected day (Schwartz *et al.*, 1980). Thus, the SCN appears to be the site of an important circadian oscillator, coupled to surrounding brain structures primarily by neural connections.

Destruction of sparrows' SCN disrupts free-running locomotion, producing arrhythmia. The birds still entrain to LD cycles, so the results approximate those for pinealectomy (Takahashi and Menaker, 1979). These authors favor the idea that a third oscillator, with input from light receptors, can entrain locomotion.

While ablation helps identify a candidate structure, it cannot reveal the timing mechanism. One approach to the latter is to study the metabolism of brain structures. The autoradiographic 2-deoxy-D-glucose method has been used to measure rhythmic glucose utilization in the intact SCN of rats (Schwartz *et al.*, 1980). Glucose use should reflect the functional activity of the SCN (Schwartz and Gainer, 1977), since brain structures depend heavily upon glucose for energy. Animals were injected intravenously with radiolabeled 2-deoxy-glucose and killed 45 min later. Frozen sections of the brain were made, autoradiographs prepared, and glucose utilization calculated. Under an LD cycle, the SCN glucose utilization was rhythmic. It was highest during the light portion of the cycle, coincident with highest rates of neuronal firing. The rhythmic pattern persisted in prolonged darkness (Fig. 8) and after bilateral enucleation, showing that the rhythm is endogenous to the SCN and not simply driven by light input. Further questions about SCN mechanisms can be approached by refinements in this method. Some obvious questions are what proportions of the energy are used for ion pumping, macromolecular synthesis, and transmitter release.

CELLULAR AND MOLECULAR MECHANISMS

Genetic selection

Genetic analysis and mutants have enabled investigators of complex biological systems to deal with a specific facet of a system, rather than analyze the total system's responses. This approach has been useful in dissecting the circadian-clock system. Two complementary approaches have been used. One involves isolating and characterizing clock mutants and attempting to analyze the primary gene products biochemically. The other approach is screening known biochemical mutants for circadian-clock abnormalities. This should identify important mechanisms in the circadian oscillator.

Several clock mutants are known. In *Drosophila*, a number have been isolated after induced mutagenesis. Konopka and Benzer (1971) used ethyl methane sulfonate (EMS) to mutagenize male *D. melanogaster.* These males were mated with attached-X females to produce male progeny with identically mutagenized X chromosomes. Screening these flies for abnormal rhythms in eclosion and activity led to identification of three clock mutants, one with a long period, per^l, one with a

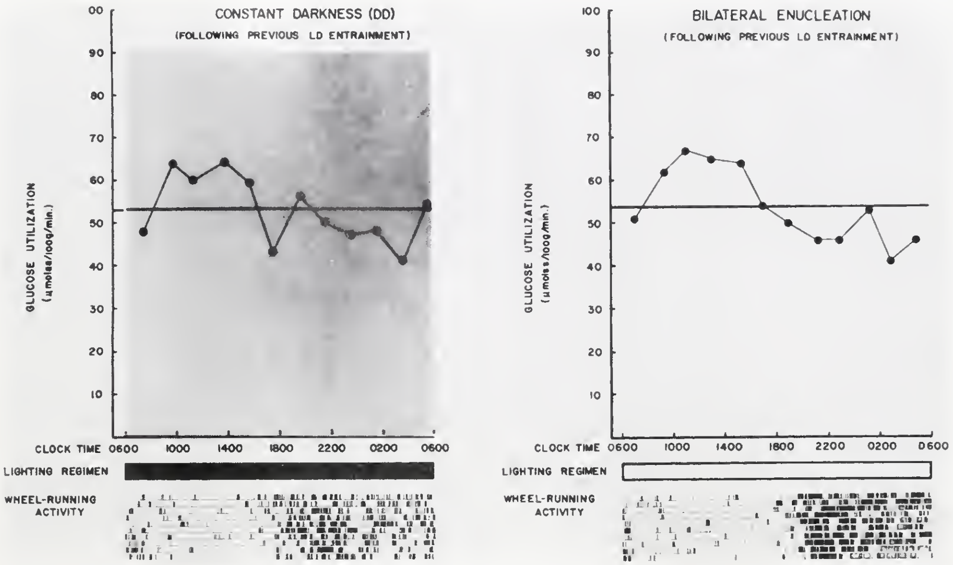


FIGURE 8. Glucose utilization by the SCN in normal rats in constant darkness (left) and bilaterally enucleated (right) rats in constant light. In both cases the utilization was rhythmic with highest points during projected day, even though locomotor activity is highest (bottom records) during projected night. Each point on the upper graphs represent one animal, except for 1100. From Schwartz *et al.*, 1980.

short period, per^s, and one that was aperiodic, per^0. All three mutants mapped to approximately the same location on the X-chromosome. Complementation tests showed that two are recessive to wild type, since heterzygotes that contain one normal and one mutant X-chromosome have nearly normal circadian rhythms. Gynandromorphs with clock-mutant X-chromosomes have been constructed (Hotta and Benzer, 1972) and behavioral mapping of these mosaic flies showed that the clock is in the brain, and that it probably exists independently on each side of the symmetrical brain.

In addition to altered periodicity, the mutants have altered responses to phase resetting after light pulses. Normal *D. melanogaster* have a small-amplitude phase-response curve (light shifts the oscillator in small steps). But the short period mutant has a phase-response curve with a larger amplitude, and a smaller portion of the cycle where light has no effect. So the mutations affect not only the period, but also other aspects of the circadian clock organization (Konopka, 1979).

Genetic clock mutants obtained in *Drosophila pseudoobscura* (Pittendrigh, 1974) have been studied by testing the clock control of the eclosion of pupae to adults. EMS mutagenesis similar to that performed in *D. melanogaster* produced five mutants that fall into two groups. Group 1 shows weak periodicity in a light-dark cycle and no periodicity in constant darkness. Group 2 shows no periodicity in either regimen. Mutants within each group do not complement one another, but partial complementation is obtained for heterozygotic flies with group 1 mutations on one X-chromosome and group 2 mutations on the other X-chromosome. They are periodic in constant darkness, but the phase and pattern of their rhythms are altered.

Since the *D. melanogaster* mutants map to the brain in genetic mosaics, it should be possible to transplant a fly brain to a mutant fly and have the transplant's circadian rhythm expressed, provided the clock's output becomes coupled to the locomotor apparatus. This result has been obtained. Handler and Konopka (1979) transplanted brains from short-period mutant (per^s) animals to the abdomens of aperiodic-mutant (per^0) host flies. The hosts then expressed short-period rhythms. These experiments show that the clock is in the transplanted brain and is coupled to the locomotory control mechanism by a humoral agent, since no neural attachments were restored. Recent evidence suggests that neurosecretory cells in the brain are concerned with the clock, because these cells are located atypically in the aperiodic mutant (Konopka and Wells, 1980) and in the *D. pseudoobscura* mutants. The aperiodic mutation has a significantly larger percentage of neurosecretory cells at the top edge of the brain. However, normal morphological distribution of neurosecretory cells does not guarantee normal circadian rhythmicity, since a few per^0 individuals with normal cell morphology still show aperiodic locomotor activity. These neurosecretory cells probably are involved in the circadian clock system, perhaps releasing a humoral substance controlling locomotor activity (Konopka and Wells, 1980).

The photosynthetic flagellate *Chlamydomonas* has rhythms in phototaxis and growth. Most wild-type strains have normal circadian periods, but a wild-type strain was found with a period 3 h shorter than average (Bruce, 1976), showing that variation does occur in wild populations. Mutagenesis of normal strains with nitrosoguanadine produced long-period mutants (Bruce, 1972). The mutants behave like single-gene mutations, since pairwise crosses between mutants resulted in recombinant forms from all crosses. Double mutant recombinants indicate additive effects of the genes: double mutants' periods are lengthened by twice the single mutant increase of 2.5–4 h (Bruce, 1976). Analysis of three of these mutants, per-1, per-2, and per-4, demonstrated recessive, dominant, and incompletely dominant modes of inheritance (Bruce and Bruce, 1978).

Neurospora shows a circadian rhythm in the periodic formation of conidia as the cultures grow along the length of cylindical growth tubes. The *band* strain produces conidia at intervals of 21.5 h in such "race" tubes. Screening of colonies grown from nitrosoguanadine-treated conidia revealed a variety of mutants with altered periods (Feldman and Hoyle, 1973, 1976). Seven of these are single-gene mutants at the same genetic locus, designated "frequency" (*frq*), on linkage group IIIR. Three have periods shorter than normal (down to 18 h) and four have longer periods (up to 29 h). Mutants at the *frq* locus share important properties. Light pulses evoke responses that suggest that their subjective day part of the cycle is altered but their subjective night portion is unaffected. Mutants are incompletely dominant to wild type, as heterokaryons containing mutant and wild-type nuclei have circadian periodicities intermediate in length. The changes in period are proportional to the percentage of mutant nuclei—a gene dose effect. Each mutant differs from wild type in period length by 2.5, 5.0, or 7.5 h, as if alterations in the period occur in discrete steps (Feldman *et al.*, 1979).

While mutations at the *frq* locus of *Neurospora* are mutations of a single gene that does not affect other characteristics (*e.g.*, growth), mutations at five other genetic loci also can alter the period. No unique locus determines period length. Construction of double or triple mutants shows that mutations' effects are often cumulative—one triple mutant had a period length of 38.5 h (Feldman *et al.*, 1979).

A summary of circadian-clock period mutants (Table I) shows that mutations are not consistently dominant or recessive, that they can occur at different loci in the same organism, and they can greatly decrease or increase the period length,

TABLE I

Circadian clock mutants

Organism	Strain	Period	Linkage	Dominance	Reference
Drosophila melanogaster	per^s	19	X chromosome	Incomplete	Konopka & Benzer (1971)
	per^1	28	X chromosome	Recessive	
	per^0	aperiodic	X chromosome	Recessive	
D. pseudobscura					Pittendrigh (1974)
	group 1	aperiodic	X chromosome	Incomplete	
	group 2	aperiodic	X chromosome	Incomplete	
Chlamydomonas					Bruce & Bruce (1978)
	per 1	28	—	Dominant	
	per 2	27	—	Recessive	
	per 4	28	—	Incomplete	
Neurospora					Feldman *et al.* (1979)
	frq 1	16.5	VIIR	Incomplete	
	frq 2	19.3	VIIR	Incomplete	
	frq 3	24.0	VIIR	Incomplete	
	frq 4	19.3	VIIR	Incomplete	
	frq 6	19.2	VIIR	—	
	frq 7	29.0	VIIR	Incomplete	
	frq 8	29.0	VIIR	—	
	prd	25.8	IIIC	Recessive	
	chr	23.5	VIL	—	
	IV 2	25.5	V R	—	
	IV 4	25.1	IC	—	
	V 8	18	?	—	

perhaps in discrete steps. The circadian clock mechanism is complicated and appears to require many gene products for proper functioning.

Screening biochemical mutants for clock abnormalities also has been productive. Crossing the *band* strain of *Neurospora* with an oligomycin-resistant strain shortened the normal 21.5 h period to 18–19 h and slowed growth by 30% (Dieckman and Brody, 1980). The oligomycin-resistance locus maps very close to the *frq* clock-period locus. The resistance itself may be due to a change in a single mitochondrial protein. However, the real significance of this finding is yet to be determined, because oligomycin does not phase shift the clock in *Neurospora* and the oligomycin-resistance mutation may include more map units than a single peptide.

Another way that screening biochemical mutants can be useful is shown by a recent result in *Neurospora* (Nakashima *et al.*, 1980, 1981a). Cycloheximide-resistant strains crossed with *band* produced mutants that had normal rhythms but, unlike normal *Neurospora*, could not be phase shifted by cycloheximide. This showed that cycloheximide phase shifts the rhythm by inhibiting protein synthesis, and not by some side-effect of the inhibitor molecule.

Models

A plausible model of the circadian clock, the membrane model (Njus *et al.*, 1974; Njus *et al.*, 1976), brings together much of the biochemical evidence and mathematical descriptions of the circadian oscillator. Two propositions are implicit in the model: (1) the clock is found in a single cell, and (2) all eucaryotes have a clock based on the same principles. The model is compatible with the prevailing view that the clock is a limit-cycle oscillator (Fig. 9, and Tyson *et al.*, 1976). It identifies the ion gradient and ion transport activity of the cell as the state (dependent) variables in this oscillator. The ion gradient would develop across a membrane (unspecified for lack of evidence) *e.g.* of an organelle, such as the endoplasmic reticulum, or the plasma membrane. The oscillator's trajectory in time is traced out in the phase plane as a stable-limit cycle (Fig. 9) in which the X axis is ion concentration and the Y axis is ion transport activity. Each point on the limit cycle, quantified by a value for each variable, corresponds to a specific phase (or circadian time: 0, 6, 12, 18 h, etc.). Phase shifting could entrain the oscillator to a forcing LD cycle. In the model, light reduces the ion gradient (X axis) instantaneously to a new value dependent upon the strength of the light pulse (*e.g.*, dotted lines at CT 10 and 18). The oscillator would resume motion from that point and eventually would return to the stable limit cycle, but with a permanent phase shift—advanced or delayed depending on the time in the cycle when the pulse was given. The same action would be expected of any agent (*e.g.*, chemical pulse) that altered the ion gradient.

To explain the generation of slow oscillations, suitable for circadian periods, Njus *et al.* suggested that the arrangement of membrane particles determines transport activity, and that rearranging these components was a slow process requiring lateral diffusion in the lipid of the membrane. The adjustment of the saturation of membrane lipids in response to temperature changes was seen as a possible explanation for temperature compensation of the period shown by circadian clocks. Thus, the membrane model offers explanations for most of the features of circadian clocks.

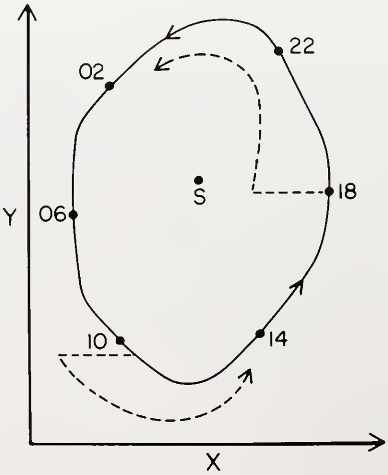


FIGURE 9. Limit cycle oscillator, depicting the relationship between the state variables X and Y in the phase plane. Time (or phase) in the circadian cycle is given by the circadian time points, 02, 06, 10, 14, 18, 22, which are successively encountered with counterclockwise motion around the stable limit cycle (solid line). The state variables in the membrane model are X = ion concentration and Y = ion transport, but other variables could be appropriate in other models. Perturbations of the oscillator with natural time givers or chemical pulses are shown here (CT 10 and CT 18) to reduce the X variable instantly, pushing the oscillator off the limit cycle. The oscillation (dotted lines) then approaches the limit cycle again but with a permanent phase advance (CT 18) or phase delay (CT 10). The amount of shift depends upon the strength of the perturbing stimulus. If the oscillator is driven to the singularity point(s) arrhythmia results until the oscillator is moved off the singularity. Adapted from Njus *et al.*, 1976, and Tyson *et al.*, 1976.

The membrane model is not explicit on many points (*e.g.* which ions, which membranes). But it focuses attention on the evidence for the involvement of ions and ion transport in the clock. High potassium pulses phase shift the rhythms of organisms as diverse as *Aplysia* (Eskin, 1972) and bean plants (Bünning and Moser, 1973). Lithium lengthens the period of rhythms (Engelmann, 1973). The ionophores valinomycin (Sweeney, 1976) and A23187 (Eskin and Corrent, 1977) phase shift rhythms, and ethanol is notorious for its period-lengthening and phase-shifting effects. Sweeney (1976) and Eskin (1979) summarized the evidence for the involvement of ions and membranes in the clock.

Progress in gathering additional evidence to support the membrane model has been slow, and evidence in favor of the involvement of protein synthesis in the clock has accumulated rapidly. This latter evidence will be reviewed in a later section, but it should be noted here that ion gradients and ion transport might be controlled by alternating synthesis and degradation of conductance or transport channel proteins (Njus *et al.*, 1976) or of transport enzymes. Also, membranes may provide a surface for maintaining the proper geometrical arrangement of ribosomal units during protein synthesis (Palade, 1975).

Other models with varying degrees of utility have been proposed. One was the chronon model of Ehret and Trucco (1967), in which messenger RNA would be sequentially transcribed from a clock gene. Scant evidence supports this model. One model offered frequently is a feedback-relaxation oscillator model, exemplified by that of Benson and Jacklet (1977b) for the *Aplysia* eye rhythm. In this model a chemical substance(s) (now thought to be specific proteins) is synthesized and accumulates to a certain level. Further synthesis is controlled by comparing the existing level to a reference level. If the reference level is exceeded, synthesis is switched off, resuming again when the level falls below the reference. Light can adjust the reference level and thereby phase shift the oscillator. This feature is similar to light-induced phase shifting in the membrane model, where light acts to reduce an ion gradient. Blocking synthesis also can cause phase shifts in the model. The phase shifts in the model resulting from light or synthesis inhibition differ, as found experimentally.

The coupled translation-membrane model proposed by Schweiger and Schweiger (1977) features essential membrane proteins (synthesized each cycle) and membrane assembly as the oscillator's central components. The essential proteins, assembled into membranes, change the characteristics of the membranes. Regulation of protein synthesis (switching on and off) depends upon thresholds in the loading of proteins into the membrane. This is analogous to the reference level and switching in the Benson and Jacklet model. Neither of these models offers a concise outline of the oscillator.

A biochemical feedback model involving c-AMP has been proposed by Cummings (1975), but more recent evidence on the involvement of c-AMP does not support this concept: *Neurospora* mutants deficient in adenylate cyclase have perfectly good rhythms (Feldman *et al.*, 1979).

Macromolecular synthesis, proteins, and RNA

Protein synthesis on the eucaryotic ribosome (80S) is now viewed as an important aspect of the circadian timing mechanism, although some early experiments examining this proposition were equivocal (Sweeney *et al.*, 1967). Good evidence supporting the involvement of protein synthesis was obtained by Feldman (1967), who showed lengthening of the period of the biological clock in *Euglena* by step applications of cycloheximide, an inhibitor of protein synthesis. The effect was proportional to the degree of inhibition of protein synthesis. This work stood alone for several years before other positive results were obtained.

According to the criteria discussed at the Dahlem Conference (Tyson *et al.*, 1976), a chemical agent, such as a protein synthesis inhibitor, affects the basic clock mechanism if the period of the clock is altered by the continuous presence of the agent or if the phase of the rhythm is shifted when the agent is applied as a brief pulse (*i.e.*, short compared to the length of the cycle, *e.g.*, 4–6 h). The continuous application, or step experiment, is believed to affect a parameter of the clock such as the rate of substrate utilization. The step experiment should give results proportional to the dose of the chemical being tested. A threshold concentration should be apparent. The pulse experiments are thought to act on a state variable (dependent variable that describes the oscillation) if phase shifts of the rhythm are obtained. Again, a threshold concentration should be definable and the size of the phase shift should be dose dependent. The size and direction of the phase shift also should depend upon the phase of the cycle when the pulse was given—that is, phase shifts should be phase dependent.

In an ideal situation, where the parameters and state variables of an oscillator are known, one should be able to accurately predict the effects of chemicals used in step and pulse experiments. However, the parameters and state variables of the circadian oscillator are unknown, and any distinction between parameters, state variables, or just hands of the clock is model-dependent. For example, a simple model may have only two state variables, such as ion concentration and ion transport in the membrane model. A more complete description might include additional state variables. But despite such uncertainties about their interpretation, the step and pulse experiments remain good approaches to probing the clock mechanism.

Most studies of the oscillator's protein synthesis requirement have used inhibitors of protein synthesis in step and pulse experiments. Both experiments have been made on the *Aplysia* eye rhythm. The results serve as examples here. Anisomycin, a potent protein-synthesis inhibitor that binds to the 60S subunit of the eucaryotic ribosome (Grollman, 1967), was applied in increasing dosage in a step experiment (Jacklet, 1980a). The results are shown in Figure 10. The threshold concentration for lengthening the period of the rhythm is about 10^{-8} *M*. This concentration inhibits protein synthesis by about 10% (Grollman, 1967). At 10^{-7} *M* (inhibition about 50%) the period is lengthened to more than 30 h. At 10^{-6} *M* (about 90% inhibition) the rhythm is immediately suppressed; the CAP activity is continuous, but lacks its circadian periodicity. This is a specific effect on the clock control, as the anisomycin does not seem to affect the CAP-generating mechanism. Lack of effect on neuronal membrane function was independently shown by Schwartz *et al.* (1971).

Pulses of anisomycin at 10^{-6} *M* permanently phase shift *Aplysia* eye rhythm, as shown in Figure 11. Delays (top panel) or advances (lower) are obtained depending upon the phase of the rhythm at which the pulses are applied. The rhythm returns to its normal periodicity after the pulse, showing that the inhibitor's effects on the clock are reversible, as are the effects of anisomycin on protein synthesis (Grollman, 1967). Transient differences in the amount of the phase shift often occur in the first cycle after the pulse, but a stable permanent shift is established by the third cycle. These differences were particularly noticeable at some circadian phases, showing a differential sensitivity of the oscillator to the perturbation.

Applying anisomycin pulses at different phases of the circadian cycle causes systematic differences in the direction and magnitude of the phase shifts, just as

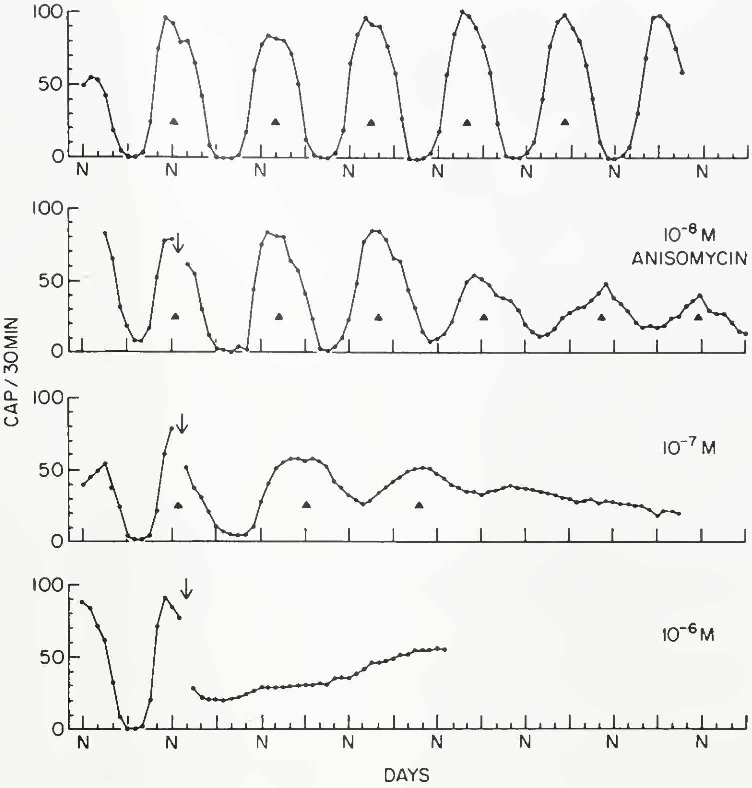


FIGURE 10. CAP frequency rhythms of *Aplysia* during the continuous presence, added at arrows, of anisomycin at 3 concentrations. The top is a control eye at 15°C in darkness, exhibiting periods of 26.5, 26.5, 26.8, 27, and 27 h. Periods at 10^{-8} *M* are 27.5, 27.5, 28.5, 32, and 26.5. Periods at 10^{-7} *M* are 35 and 31 h. The inhibitor suppressed the rhythm completely at 10^{-6} *M*, but the CAP activity continued. Successive noons (N) are the time axis. Triangles are calculated centroid points. From Jacklet, 1980b.

is expected for perturbations of a state variable (Tyson *et al.*, 1976). The phase shifts plotted against circadian time in the cycle resulted in a PRC that indicates the agent's action on the basic timing mechanism. Figure 12 shows the PRC for anisomycin pulses. Greatest delays are at CT 0–3; advances are at CT 5–7. This apparent change in sensitivity to an inhibitor suggests that proteins necessary for clock timing are synthesized at specific times (Karakashian and Schweiger, 1976b). However, this proposition has not been tested, as it is not known which newly synthesized proteins are involved in the clock mechanism and which ones might be driven by the clock. Thus, a resolution must await the identification of specific clock proteins.

In addition to *Euglena* and *Aplysia* eye, other preparations have supplied strong evidence to support involvement of protein synthesis in the oscillator. The rhythm in *Acetabularia* is phase shifted by three inhibitors: puromycin, cycloheximide, and anisomycin (Karakashian and Schweiger, 1976b). Using a liquid culture technique for *Neurospora*, Nakashima *et al.* (1980, 1981b) demonstrated phase-dependent phase shifting with cycloheximide. Even in *Gonyaulax*, where such experiments are difficult, there is positive evidence for phase shifting with cycloheximide (Walz

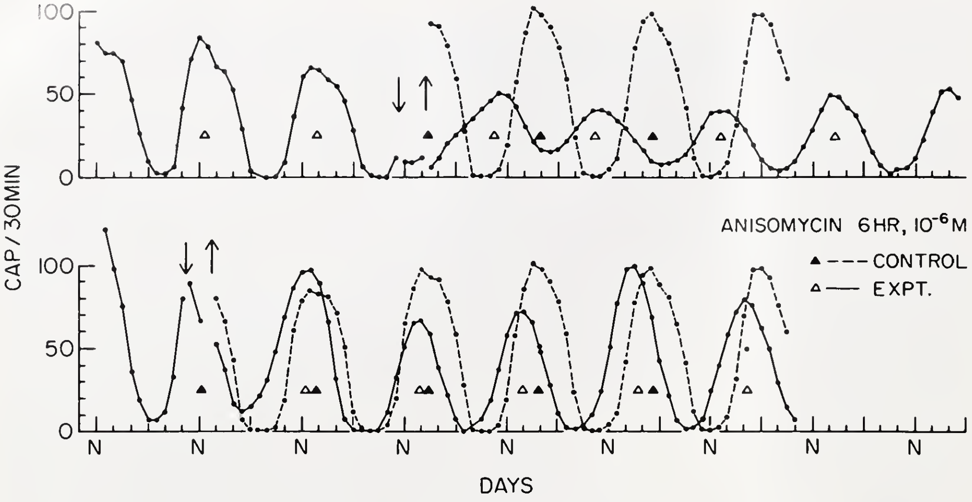


FIGURE 11. Phase shifts of the CAP frequency rhythms of *Aplysia* by 6 h pulses of anisomycin (10^{-6} *M*). The upper graph shows a phase delay when the pulse was given at CT 3 and the bottom graph shows a phase advance when the pulse was given at CT 5. Solid lines are experiment eyes and dotted lines are controls. Triangles are calculated centroid points. From Jacklet, 1980b.

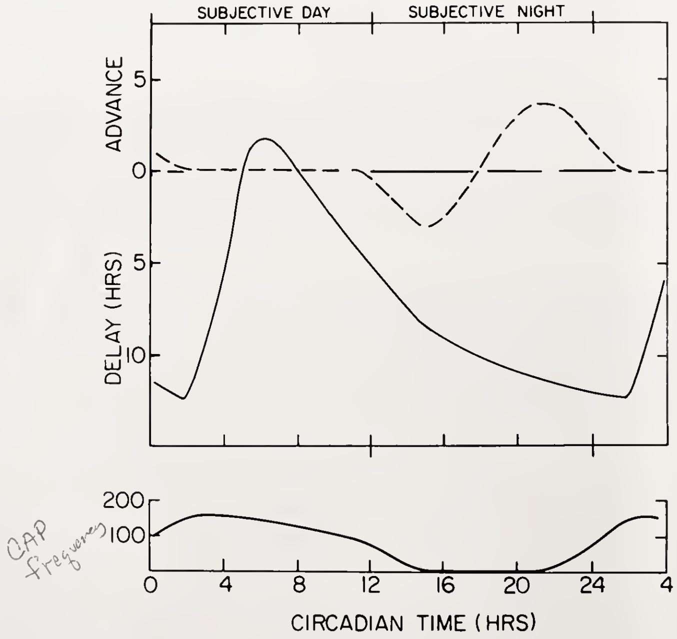


FIGURE 12. Phase response curves (PRC) for anisomycin (solid lines) and light (dotted lines) for the *Aplysia* eye rhythm. The time scale is circadian time divided into subjective day (00–12) and subjective night (12–24). The transition from phase delays to phase advances occurs about CT 18 for light pulses but at about CT 5 for anisomycin. The bottom of the figure shows the CAP frequency rhythm for phase reference. Adapted from Jacklet, 1977 and 1978.

and Sweeney, 1979; Dunlap *et al.*, 1980). The rhythm of ultrastructural changes in these cells also was phase shifted by cycloheximide (Rensing *et al.*, 1980).

Where the PRC has been extensively studied, it appears to change in a regular way in response to protein synthesis inhibitors at different concentrations. Phase shifts are smaller and have different shapes with lower concentrations. But the position of the PRC on the circadian time axis is relatively fixed (Walz and Sweeney, 1979; Dunlap *et al.*, 1980). This is also true of light-induced phase shifts. Insight into the clock may be gained from similarities and differences in PRCs evoked by various agents. The anisomycin PRC and the light PRC in *Aplysia* clearly differ, as shown in Figure 12. One may conclude that light and anisomycin act on different phases and probably different components of the clock mechanism. This prediction, indeed, follows from the model of Benson and Jacklet (1977b), where the phase-dependent effects of light, which acts on a reference level, should be different from the effects of anisomycin, which acts on synthesis.

However, in the *Gonyaulax* rhythm, the inhibitor cycloheximide may have two actions, an early one on ion distribution and a later one on protein synthesis (Walz and Sweeney, 1979). Also, the PRC is nearly identical to the PRC for light pulses. This points out the dangers of generalizing from one organism to others.

Other protein synthesis inhibitors, such as cycloheximide and puromycin, cause phase shifts of the *Aplysia* eye clock (Rothman and Strumwasser, 1976). The PRC they constructed for puromycin was similar to the one for anisomycin. These puromycin experiments were repeated using an organ culture procedure. They yielded a PRC nearly identical in shape and time position (but smaller in magnitude) to the anisomycin PRC (Lotshaw and Jacklet, 1980). This supports the notion that similar inhibitors should cause similar clock perturbations. Some other agents provoke PRCs in *Aplysia* similar to the anisomycin PRC. These include the metabolic inhibitors NaCN, DNP, the calcium ionophore A23187 (Eskin and Corrent, 1977); and low temperature (Deuser, 1979; Benson and Jacklet, 1977a) which yield PRCs with similar time positions but smaller magnitudes. The general metabolic inhibitors seem to mimic the more specific action of protein synthesis inhibitors, which act on a synthetic process that has high energy demands.

Inhibitors may have side effects. Therefore, it is important to show that any side effects are minimal and that protein synthesis is actually inhibited at the inhibitor concentration where clock effects are observed. In the *Acetabularia* experiments (Karakashian and Schweiger, 1976a) protein synthesis was about 50% inhibited at the concentration effective for phase shifting. In the *Neurospora* experiments (Nakashima *et al.*, 1980, 1981b) synthesis was reduced by 65%. In *Aplysia* 50% inhibition by puromycin was effective in phase shifting (Rothman and Strumwasser, 1976) and 80–90% inhibition with anisomycin was observed at the concentration (10^{-6} M) that affected phase-shifting (Jacklet, 1977). Thus, in a good proportion of the tests, the concentration of inhibitor that causes 50% or more inhibition also produces phase shifts of the circadian clock. Inhibitors of procaryotic protein synthesis are uniformly without effect on the clock (Karakashian and Schweiger, 1976b), implicating the eucaryotic ribosome as an important part of the clock. This is consistent with the lack of circadian rhythms in procaryotes.

However, inhibition and phase shifting could be parallel events, not necessarily causally linked. Side effects as well as protein-synthesis inhibition might be dose dependent. Two experiments strongly support a causal link between protein-synthesis inhibition and phase shifting the circadian clock. In the first, Jacklet (1980b) tested derivatives of the inhibitor anisomycin that are very similar to the parent molecule but do not inhibit protein synthesis. Only the active inhibitor molecules caused phase shifts. The inactive derivatives were completely innocuous. In the second experiment, Nakashima *et al.* (1980, 1981a), used cycloheximide-resistant *Neurospora* mutants. They demonstrated that cycloheximide is ineffective in phase-shifting those mutants, but quite effective on wild-type *Neurospora.* Therefore, the insensitivity of the ribosomes of the mutants to cycloheximide confers immunity to the inhibitor and prevents phase shifting.

Karakashian and Schweiger (1976c) noted in *Acetabularia* a difference in cycloheximide sensitivity at different temperatures. The sensitivity shifted to a different circadian phase at a different (20° vs. 25°C) physiological temperature. Since the clock is temperature compensated, this unexpected result suggested that protein synthesis was independent of the central clock mechanism. This inconsistency remains for *Acetabularia.* However, tests of other rhythms showed that the effects of inhibitors are nearly identical at different physiological temperatures, and therefore the effect is temperature compensated, in *Aplysia* (Jacklet, 1980a), *Gonyaulax* (Dunlap *et al.*, 1980), and *Neurospora* (Nakashima *et al.*, 1980).

If protein synthesis is important, then RNA synthesis could be, too, since DNA codes for RNA and RNA codes for protein. The importance of DNA synthesis has been tested in *Gonyaulax* by applying the inhibitor mitomycin C (Karakashian and Hastings, 1963). This had no effect on the circadian clock. RNA synthesis was tested using actinomycin D. It had weak effects on phase shifting the *Gonyaulax* glow rhythm and blocked rhythmicity in *Acetabularia* (Sweeney *et al.*, 1967) and in the *Aplysia* eye (Rothman and Strumwasser, 1977). The strongest evidence against RNA synthesis in clock timing is the observation of strong rhythms in enucleated *Acetabularia* (Sweeney and Haxo, 1961) and the failure of rifampicin (Vanden Driessche *et al.*, 1970) to block the rhythm in enucleated cells.

Ionizing radiation (X-rays) has been used to selectively block the circadian rhythm in the *Aplysia* eye, without altering membrane functions (Woolum and Strumwasser, 1980). Their results suggest that the eye contains a number of circadian oscillators, most of them near the optic nerve. The most likely targets of the X-rays, and therefore the elements of importance for the circadian clock, are nucleic acids. The appearance of the eye activity after irradiation is similar to the activity shown in Figure 10 after step treatment with 10^{-6} *M* anisomycin.

Most of the RNA-synthesis inhibitor studies suffer from a lack of actual measurements of the RNA synthesis. Side effects of the inhibitors may be suspect. Also, the drugs used, such as antinomycin D, are not readily reversible, making it impossible to study the periodicity or phase shift of the rhythm after treatment. A reversible RNA synthesis inhibitor with minimal side effects, and measurement of the actual RNA synthesis rates, are needed to completely resolve whether RNA synthesis is involved in the circadian clock. The evidence to date favors the conclusion that RNA synthesis is not part of the basic clock (Sargent *et al.*, 1976), but it seems likely that the amount of RNA available could readily influence the clock timing generated by protein synthesis on the eucaryotic ribosome.

Acknowledgment

This work was supported in part by National Science Foundation grant BNS 11154. I thank Alice Jacklet for helpful comments, Thea Hotaling for assistance, and Robert Speck for figure production.

LITERATURE CITED

Aréchiga, H., A. Huberman, and E. Naylor. 1974. Hormonal Modulation of circadian activity in *Carcinus maenas* (L). *Proc. R. Soc. Lond. B. Biol. Sci.* **187:** 299–313.

Aréchiga, H., and C. Wiersma. 1969. Circadian rhythm of responsiveness in crayfish visual units. *J. Neurobiol.* **1:** 71–85.

Aréchiga, H., J. Williams, R. Pullin, and E. Naylor. 1979. Cross-sensitivity to neuro-depressing hormone and its effect on locomotor rhythmicity in two different groups of crustaceans. *Gen. Comp. Endocrinol.* **37:** 350–357.

Aschoff, J. 1965. Response curves in circadian periodicity. Pp. 95–111 in J. Aschoff, Ed., *Circadian Clocks.* North-Holland Publishing Co., Amsterdam.

Aschoff, J., and R. Wever. 1976. Human circadian rhythms: a multioscillator system. *Fed. Proc.* **35:** 2326–2332.

Barlow, R., Jr., S. Bolanowski, Jr. and M. Brachman. 1977. Efferent optic nerve fibers mediate circadian rhythms in the *Limulus* eye. *Science* **197:** 86–89.

Barlow, R. Jr., S. Chamberlain, and J. Levinson. 1980. *Limulus* brain modulates the structure and function of the lateral eye. *Science* **210:** 1037–1039.

Beiswanger, C., and J. Jacklet. 1975. *In vitro* tests for a circadian rhythm in the electrical activity of a single neuron in *Aplysia californica. J. Comp. Physiol.* **103:** 19–37.

Benson, J., and J. Jacklet. 1977a. Circadian rhythm of output from neurons in the eye of *Aplysia.* I. Effects of deuterium oxide and temperature. *J. Exp. Biol.* **70:** 151–166.

Benson, J., and J. Jacklet. 1977b. Circadian rhythm of output from neurons in the eye of *Aplysia.* IV. A model of the clock: differential sensitivity to light and low temperature pulses. *J. Exp. Biol.* **70:** 195–211.

Binkley, S., J. Riebman, and K. Reilly. 1978. The pineal gland: a biological clock *in vitro. Science* **202:** 1198–1201.

Block, G. 1979. Circadian rhythm in the eye of *Aplysia* recorded *in vivo. Soc. Neurosci. Abst.* **5:** 242.

Block, G., and T. Page. 1978. Circadian pacemakers in the nervous system. *Ann. Rev. Neurosci.* **1:** 19–34.

Block, G., and M. Roberts. 1980. A circadian rhythm in the isolated eye of *Bursatella. Soc. Neurosci. Abst.* **6:** 706. Also *J. Comp. Physiol.*, in press.

Brady, J. 1969. How are insect rhythms controlled? *Nature* **223:** 781–784.

Bruce, V. 1972. Mutants of the biological clock in *Chlamydomonas reinhardi. Genetics* **70:** 537–548.

Bruce, V. 1976. Clock mutants. Pp. 339–351 in J. Hastings and H. Schweiger, Eds., *The Molecular Basis of Circadian Rhythms.* Dahlem Konferenzen, Berlin.

Bruce, V., and N. Bruce. 1978. Diploids of clock mutants of *Chlamydomonas reinhardi. Genetics* **89:** 225–233.

Bünning, E., and I. Moser. 1973. Light-induced phase shift of circadian leaf movements of *Phaseolus*: comparison with the effects of potassium and of ethyl alcohol. *Proc. Nat. Acad. Sci. U. S. A.* **70:** 3387.

Chamberlain, S., and R. Barlow, Jr. 1979. Light and efferent activity control rhabdom turnover in *Limulus* photoreceptors. *Science* **206:** 361–363.

Corrent, G., D. McAdoo, and A. Eskin. 1978. Seratonin shifts the phase of the circadian rhythm from the *Aplysia* eye. *Science* **202:** 977–979.

Cummings, F. W. 1975. A biochemical model of the circadian clock. *J. Theor. Biol.* **55:** 455–470.

Darwin, Charles. 1881. P. 25 in *The formation of vegetable mould, through the action of worms, with observations on their habits.* John Murray, Albemerle St., London.

De Mairan, J. 1729. P. 35 in *Observations Botanique.* Paris, Histoire de L'Academie Rogate des Sciences.

Deguchi, T. 1979. A circadian oscillator in cultured cells of chicken pineal gland. *Nature* **282:** 94–96.

Deuser, M. 1979. The effects of cold temperature and the substitution of propionate for chloride on the circadian rhythm of action potential in the eye of *Aplysia.* M.S. Thesis, State University of New York at Albany. 95 pp.

Dieckman, C. and S. Brody. 1980. Circadian rhythms in *Neurospora crassa*: oligomycin-resistant mutations affect periodicity. *Science* **207:** 896–898.

Dunlap, J., W. Taylor, and J. Hastings. 1980. The effect of protein synthesis inhibitors on the *Gonyaulax* clock: phase-shifting effects of cycloheximide. *J. Comp. Physiol. B.* **138:** 1–8.

Ehret, C., and E. Trucco. 1967. Molecular models for the circadian clock. I. The chronon concept. *J. Theoret. Biol.* **15:** 240–262.

Engelman, W. 1973. A slowing down of circadian rhythms by lithium ions. *Z. Naturforsh.* **28:** 733–736.

Eskin, A. 1972. Phase shifting a circadian rhythm in the eye of *Aplysia* by depolarizing high potassium pulses. *J. Comp. Physiol.* **80:** 353–376.

ESKIN, A. 1977. Neurophysiological mechanisms involved in photo-entrainment of the circadian rhythm from the *Aplysia* eye. *J. Neurobiol.* **8:** 273–299.

ESKIN, A. 1979. Circadian systems of the *Aplysia* eye: properties of the pacemaker and mechanisms of its entrainment. *Fed. Proc.* **38:** 2573–2579.

ESKIN, A., AND G. CORRENT. 1977. Effects of divalent cations and metabolic poisons on the circadian rhythm of the *Aplysia* eye. *J. Comp. Physiol.* **117:** 1–21.

ESKIN, A., AND E. HARCOMBE. 1977. Eye of *Navanax*: optic activity, circadian rhythm and morphology. *Comp. Biochem. Physiol.* **57A:** 443–449.

FELDMAN, J. 1967. Lengthening the period of the biological clock in *Euglena* by cycloheximide, an inhibitor of protein synthesis. *Proc. Nat. Acad. Sci. U. S. A.* **57:** 1080–1087.

FELDMAN, J., G. GARDNER, AND R. DENISON. 1979. Genetic analysis of the circadian clock of *Neurospora*. Pp. 57–66 in M. Suda, D. Hayaishi, and H. Nakagawa, Eds., *Biological Rhythms and Their Central Mechanism, Naito Foundation.* Elsevier/North-Holland Biomedical Press, Amsterdam.

FELDMAN, J., AND M. HOYLE. 1973. Isolation of circadian clock mutants of *Neurospora crassa. Genetics* **75:** 605–613.

FELDMAN, J., AND M. HOYLE. 1976. Complementation analysis of linked circadian clock mutants of *Neurospora crassa. Genetics* **82:** 9–17.

GROLLMAN, A. P. 1967. Inhibitors of protein biosynthesis. II. Mode of action of anisomycin. *J. Biol. Chem.* **242:** 3226–3233.

GWINNER, E. 1978. Effects of pinealectomy on circadian locomotor activity rhythms in European starlings, *Sturnis vulgaris. J. Comp. Physiol.* **126:** 123–129.

HALBERG, F. 1960. Temporal coordination of physiologic function. *Cold Spring Harbor Symp. Quant. Biol.* **25:** 289–310.

HAMM, H., AND M. MENAKER. 1980. Retinal rhythms in chicks: Circadian variations in melatonin and serotonin N-acetyltransferase activity. *Proc. Natl. Acad. Sci. U. S. A.* **77:** 4998–5002.

HANDLER, A., AND R. KONOPKA. 1979. Transplantation of a circadian pacemaker in *Drosophila. Nature* **279:** 236–238.

HARF, L., S. ARCH, AND A. ESKIN. 1976. Polypeptides secretion from the eye of *Aplysia californica. Brain Res.* **111:** 295–299.

HASTINGS, J. W. 1960. Biochemical aspects of rhythms: phase shifting by chemicals. *Cold Spring Harbor Symp. Quant. Biol.* **25:** 131–144.

HASTINGS, J. W., J. ASCHOFF, E. BÜNNING, L. EDMUNDS, K. HOFFMANN, C. PITTENDRIGH, AND A. WINFREE. 1976. Basic features group report. Pp. 49–62 in J. Hastings and H. Schweiger, Eds., *The Molecular Basis of Circadian Rhythms.* Dahlem Konferenzen, Berlin.

HASTINGS, J. W., AND H. G. SCHWEIGER, EDS. 1976. P. 461, *The Molecular Basis of Circadian Rhythms.* Dahlem Konferenzen, Berlin.

HOTTA, Y. AND S. BENZER. 1972. Mapping of behaviour in *Drosophila* mosaics. *Nature* **240:** 527–535.

HUDSON, D., AND M. LICKEY. 1980. Internal desynchronization between the identified circadian oscillators in *Aplysia. Brain Res.* **183:** 481–485.

INOUYE, S., AND H. KAWAMURA. 1979. Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc. Nat. Acad. Sci. U. S. A.* **76:** 5962–5966.

JACKLET, J. W. 1969a. Circadian rhythm of optic nerve impulses recorded in darkness from isolated eye of *Aplysia. Science* **164:** 562–563.

JACKLET, J. W. 1969b. Electrophysiological organization of the eye of *Aplysia. J. Gen. Physiol.* **53:** 21–42.

JACKLET, J. W. 1971. A circadian rhythm in optic nerve impulses from an isolated eye in darkness. Pp. 351–362 in M. Menaker, Ed., *Biochemistry.* National Academy of Sciences, Washington, D. C.

JACKLET, J. W. 1973. Neuronal population interactions in a circadian rhythm in *Aplysia.* Pp. 363–380 in J. Salánki, Ed., *Neurobiology of Invertebrates.* Hungarian Academy Sciences, Budapest.

JACKLET, J. W. 1976. Dye marking neurons in the eye of *Aplysia. Comp. Biochem. Physiol.* **55A:** 373–377.

JACKLET, J. W. 1977. Neuronal circadian rhythm: phase shifting by a protein synthesis inhibitor. *Science* **198:** 69–71.

JACKLET, J. W. 1978. The cellular mechanism of circadian clocks. *Trends Neurosci.* **1**(5): 117–119.

JACKLET, J. W. 1979. Neuronal network of the eye of *Aplysia*: intracellular and dye marking analysis. *Soc. Neurosci. Abst.* **5:** 248.

JACKLET, J. W. 1980a. Circadian rhythm from the eye of *Aplysia*: temperature compensation of the effects of protein synthesis inhibitor. *J. Exp. Biol.* **84:** 1–15.

JACKLET, J. W. 1980b. Protein synthesis requirement of the *Aplysia* circadian clock, tested by active and inactive derivatives of the inhibitor anisomycin. *J. Exp. Biol.* **85:** 33–42.

KAPLAN, E., AND R. BARLOW, JR. 1980. Circadian clock in *Limulus* brain increases response and decreases noise of retinal photoreceptors. *Nature* **286:** 393–395.

KARAKASHIAN, M., AND J. W. HASTING. 1963. The effects of inhibitors of macromolecular biosynthesis upon the persistent rhythm of luminescence in *Gonyaulax. J. Gen. Physiol.* **47:** 1–12.

KARAKASHIAN, M., AND H. SCHWEIGER. 1976a. Circadian properties of the rhythmic system in individual nucleated and enucleated cells of *Acetabularia mediterranea. Exp. Cell Res.* **97:** 366–377.

KARAKASHIAN, M., AND H. SCHWEIGER. 1976b. Evidence for a cycloheximide sensitive component in the biological clock of *Acetabularia. Exp. Cell Res.* **98:** 303–312.

KARAKASHIAN, M., AND H. G. SCHWEIGER. 1976c. Temperature dependence of cycloheximide sensitive phase of circadian cycle in *Acetabularia mediterranea. Proc. Nat. Acad. Sci. U. S. A.* **73:** 3216–3219.

KLEIN, D. 1974. Circadian rhythms in indole metabolism in the rat pineal. Pp. 509–575 in F. O. Schmitt and F. Worden, Eds., *The Neurosciences: Third Study Program*, MIT Press, Cambridge, MA.

KONOPKA, R. 1979. Genetic dissection of the *Drosophila* circadian system. *Fed. Proc.* **38:** 2602–2605.

KONOPKA, R., AND S. BENZER. 1971. Clock mutants of *Drosophila melanogaster. Proc. Nat. Acad. Sci. U. S. A.* **68:** 2112–2116.

KONOPKA, R., AND S. WELLS. 1980. *Drosophila* clock mutations affect the morphology of a brain neurosecretory cell group. *J. Neurobiol.* **11:** 411–415.

KRASNOW, R., J. DUNLAP, W. TAYLOR, AND J. W. HASTINGS. 1980. Circadian spontaneous bioluminescent glow and flashing of *Gonyaulax polyedra. J. Comp. Physiol. B.* **138:** 19–26.

LICKEY, M., G. BLOCK, D. HUDSON, AND J. SMITH. 1976. Circadian oscillators and photoreceptors in the gastropod, *Aplysia. Photochem. and Photobiol.* **23:** 253–273.

LICKEY, M., AND J. WOZNIAK. 1979. Circadian organization in *Aplysia* explored with red light, eye removal and behavioral recording. *J. Comp. Physiol.* **131:** 169–177.

LICKEY, M., J. WOZNIAK, G. BLOCK, D. HUDSON, AND G. AUGTER. 1977. The consequences of eye removal for the circadian rhythm of behavioral activity in *Aplysia. J. Comp. Physiol.* **118:** 121–143.

LOTSHAW, D., AND J. JACKLET. 1980. Neuronal circadian oscillator: rhythm in protein synthesis in the eye of *Aplysia. Soc. Neurosci. Abst.* **6:** 577.

LUBORSKY-MOORE, J., AND J. W. JACKLET. 1977. Ultrastructure of the secondary cells in the *Aplysia* eye. *J. Ultrastruct. Res.* **60**(2): 235–245.

MOORE, R. Y., AND V. B. EICHLER. 1972. Loss of circadian adrenal corticosterone rhythm following suprachiasmatic lesion in rat. *Brain Res.* **42:** 201–206.

MOORE, R. Y. AND D. KLEIN. 1974. Visual pathways and the central neural control of the circadian rhythm in pineal serotonin N-acetyltransferase activity. *Brain Res.* **71:** 17–33.

MCMURRAY, L., AND J. W. HASTINGS. 1972. No desynchronization among four circadian rhythms in the unicellular alga, *Gonyaulax polyedra. Science* **175:** 1137–1139.

NAKASHIMA, H., J. PERLMAN, AND J. FELDMAN. 1980. Evidence for protein synthesis requirement for the circadian clock for *Neurospora. Eur. J. Cell Biol.* **22:** 498.

NAKASHIMA, H., J. PERLMAN, AND J. FELDMAN. 1981a. Genetic evidence that protein synthesis is required for the circadian clock of *Neurospora. Science*, in press.

NAKASHIMA, H., J. PERLMAN, AND J. FELDMAN. 1981b. Cycloheximide-induced phase shifting of the circadian clock of *Neurospora. Am. J. Physiol.*, in press.

NJUS, D., V. D. GOOCH, D. MERGENHAGEN, F. SULZMAN, AND J. W. HASTINGS. 1976. Membranes and molecules in circadian systems. *Fed. Proc.* **35:** 2353–2357.

NJUS, D., F. SULZMAN, AND J. W. HASTINGS. 1974. Membrane model for the circadian clock. *Nature* **248:** 116–120.

PALADE, G. 1975. Intracellular aspects of the process of protein synthesis. *Science* **189:** 347–358.

PITTENDRIGH, C. S. 1974. Circadian oscillations in cells and the circadian organization of multicellular systems. Pp. 437–458 in F. O. Schmitt and F. G. Worden, Eds., *The Neurosciences: Third Study Program.* MIT Press, Cambridge, MA.

PITTENDRIGH, C. S. 1976. Circadian rhythms: what are they? Pp. 11–48 in J. Hastings and H. Schweiger, Eds., *The Molecular Basis of Circadian Rhythms.* Dahlem Konferenzen, Berlin.

PITTENDRIGH, C., AND S. DAAN. 1976a. A functional analysis of circadian pacemakers in nocturnal rodents: I. The stability and lability of spontaneous frequency. *J. Comp. Physiol. A* **106:** 223–252.

PITTENDRIGH, C. S., AND S. DAAN. 1976b. A functional analysis of circadian pacemakers in nocturnal rodents: V. Pacemakers structure: a clock for all seasons. *J. Comp. Physiol. A* **106:** 333–335.

RENSING, L., W. TAYLOR, J. DUNLAP, AND J. W. HASTINGS. 1980. The effects of protein synthesis inhibitors on the *Gonyaulax* clock. II. The effect of cycloheximide on ultrastructural parameters. *J. Comp. Physiol. B.* **138:** 9–18.

ROBERTS, S. K. 1974. Circadian rhythms in cockroaches. Effects of optic lobe lesions. *J. Comp. Physiol.* **88:** 21–30.

ROMERO, G., M. ZATZ, AND J. AXELROD. 1975. Beta-adrenergic stimulation of pineal N-acetyl transferase:adenosine 3':5'-cyclic monophosphate stimulates both RNA and protein synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **72:** 2107–2111.

ROTHMAN, B., AND F. STRUMWASSER. 1976. Phase shifting the circadian rhythm of neuronal activity in the isolated *Aplysia* eye with puromycin and cycloheximide. *J. Gen. Physiol.* **68:** 359–384.

ROTHMAN, B., AND F. STRUMWASSER. 1977. Manipulation of a neuronal circadian oscillator with inhibitors of macromolecular synthesis. *Fed. Proc.* **36:** 2050–2055.

RUSAK, B. 1979. Neural mechanisms for entrainment and generation of mammalian circadian rhythms. *Fed. Proc.* **38:** 2589–2595.

RUSAK, B., AND I. ZUCKER. 1979. Neural regulation of circadian rhythms. *Physiol. Rev.* **59:** 449–526.

SARGENT, M., I. ASHKENAZI, E. BRADBURY, V. BRUCE, C. EHRET, J. FELDMAN, M. KARAKASHIAN, R. KONOPKA, D. MERGENHAGEN, G. SCHÜTZ, H. SCHWEIGER, AND T. VANDEN DRIESSCHE. 1976. The role of genes and their expression group report. Pp. 295–310 in J. Hastings and H. Schweiger, Eds., *The Molecular Basis of Circadian Rhythms.* Dahlem Konferenzen, Berlin.

SCHWARTZ, J., V. CASTELLUCCI, AND E. KANDEL. 1971. Functioning of identified neurons and synapses in abdominal ganglion of *Aplysia* in absence of protein synthesis. *J. Neurophysiol.* **34:** 939–953.

SCHWARTZ, W. J., L. C. DAVIDSEN, AND C. B. SMITH. 1980. *In vivo* metabolic activity of a putative circadian oscillator, the rat suprachiasmatic nucleus. *J. Comp. Neurol.* **189:** 157–167.

SCHWARTZ, W. J., AND H. GAINER. 1977. Suprachiasmatic nucleus: use of ^{14}C-labeled deoxyglucose uptake as a functional marker. *Science* **197:** 1089–1091.

SCHWEIGER, H. G., AND M. SCHWEIGER. 1977. Circadian rhythms in unicellular organisms: an endeavor to explain the molecular mechanism. In G. H. Bourne and J. F. Danielli, Eds., *Internatl. Rev. Cytol.* **51:** 315–342.

SOKOLOVE, P. 1975. Localization of the cockroach optic lobe circadian pacemaker with microlesions. *Brain Res.* **87:** 13–21.

SOKOLOVE, P., AND W. LOHER. 1975. Role of eyes, optic lobes, and pars intercerebralis in locomotory and stridulatory circadian rhythms of *Teleogryllus commodus. J. Insect Physiol.* **21:** 785–799.

STEPHAN, F., AND I. ZUCKER. 1972. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc. Natl. Acad. Sci. U. S. A.* **69:** 1583–1586.

STRUMWASSER, F. 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. Pp. 442–462 in J. Aschoff, Ed., *Circadian Clocks*, North Holland Publishing Co., Amsterdam.

STRUMWASSER, F. 1973. Neural and humoral factors in the temporal organization of behavior. *The Physiologist* **16:** 9–42.

STRUMWASSER, F., R. ALVAREZ, D. VIELE, AND T. WOOLUM. 1979. Structure and function of a neuronal circadian oscillator system. Pp. 41–56 in M. Suda, D. Hayaishi and H. Nakagawa, Eds., *Biological Rhythms and their Central Mechanism, Naito Foundation.* Elsevier/North-Holland Biomedical Press, Amsterdam.

SWEENEY, B. 1960. The photosynthesis rhythm in single cells of *Gonyaulax polyedra. Cold Spring Harbor Symp. Quant. Biol.* **25:** 145–148.

SWEENEY, B. 1976. Evidence that membranes are components of circadian oscillators. Pp. 267–281 in J. Hastings and H. Schweiger, Eds., *The Molecular Basis of Circadian Rhythms.* Dahlem Konferenzen, Berlin.

SWEENEY, B. AND F. HAXO. 1961. Persistence of a photosynthetic rhythm in enucleated *Acetabularia. Science* **134:** 1361.

SWEENEY, B., C. TUFFLI, AND R. RUBIN. 1967. The circadian rhythm in photosynthesis in *Acetabularia* in the presence of actinomycin D, puromycin and chloramphenicol. *J. Gen. Physiol.* **50:** 647–659.

TAKAHASHI, J. S., H. HAMM, AND M. MENAKER. 1980. Circadian rhythms of melatonin release from individual superfused chicken pineal glands *in vitro. Proc. Natl. Acad. Sci. U. S. A.* **77:** 2319–2322.

TAKAHASHI, J., AND M. MENAKER. 1979. Brain mechanisms in avian circadian systems. Pp. 95–109 in M. Suda, D. Hayaishi, and H. Nakagawa, Eds., *Biological Rhythms and their Central Mechanism, Naito Foundation.* Elsevier/North-Holland Biomedical Press, Amsterdam.

TRUMAN, J. 1972. Physiology of Insect Rhythms. II. The silkmoth brain as the location of the biological clock controlling eclosion. *J. Comp. Physiol.* **81:** 99–114.

TRUMAN, J. 1974. Physiology of insect rhythms: IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. *J. Comp. Physiol.* **95:** 281–296.

TRUMAN, J. 1979. Interaction between abdominal ganglia during the performance of hormonally triggered behavioral programmes in moths. *J. Exp. Biol.* **83:** 239–253.

TYSON, J., S. ALIVISATOS, D. RICHTER, F. GREEN, F. SCHNIEDER, AND T. PAVLIDIS. 1976. Mathematical background group report. Pp. 85–108 in J. Hastings and H. Schweiger, Eds., *The Molecular Basis of Circadian Rhythms.* Dahlem Konferenzen, Berlin.

VANDEN DRIESSCHE, T., S. BONOTTO, AND J. BRACHET. 1970. Inability of rifampicin to inhibit circadian rhythmicity in *Acetabularia* despite inhibition of RNA synthesis. *Biochim. Biophys. Acta* **224:** 631–634.

WALZ, B., AND B. SWEENEY. 1979. Kinetics of the cycloheximide induced phase changes with biological clock in *Gonyaulax. Proc. Natl. Acad. Sci. U. S. A.* **76:** 6443–6447.

WEVER, R. 1975. The circadian mutli-oscillator system of man. *Intern. J. Chronobiol.* **3:** 19–55.

WEVER, R. 1979. *The Circadian System of Man: Experiments Under Temporal Isolation.* Springer-Verlag, New York. 271 pp.

WOOLUM, J., AND F. STRUMWASSER. 1980. The differential effects of ionizing radiation on the circadian oscillator and other functions in the eye of *Aplysia. Proc. Natl. Acad. Sci. U. S. A.* **77:** 5542–5546.

WURTMAN, R., J. AXELROD, AND D. KELLY. 1968. *The Pineal.* Academic Press. New York and London. 199 pp.

ZIMMERMAN, N. AND M. MENAKER. 1979. The pineal gland: a pacemaker within the circadian system of the house sparrow. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 999–1003.

Reference: *Biol. Bull.*, **160**: 228–239. (April, 1981)

AN ULTRASTRUCTURAL ANALYSIS OF CYTOPLASMIC LOCALIZATION IN *CHAETOPTERUS PERGAMENTACEUS*

WILLIAM R. ECKBERG

Department of Zoology, Howard University, Washington, D. C. 20059, and
Marine Biological Laboratory, Woods Hole, MA 02543

ABSTRACT

During early development of *Chaetopterus pergamentaceus*, cytoplasmic components visible by electron microscopy became segregated. The hyaloplasm, or spongy layer, composed of granular bodies surrounded by a matrix of endoplasmic reticulum and microtubules was segregated towards (1) the upper pole of the unfertilized egg, (2) the interior of the cleaving egg, (3) the superficial regions of the 16-cell stage, and (4) the ectoderm of the trochophore larva. The ectoplasm, characterized by cortical granules, surrounded the unfertilized and cleaving egg endoplasm. It was restricted to the apical end of cells at the 16-cell stage, and to the ectoderm of the trochophore larva. The endoplasm, characterized by yolk and lipid, (1) was found throughout the unfertilized egg except at the upper pole, (2) was restricted to a ring just below the cortex of the cleaving egg, (3) moved to the basal ends of blastomeres by the 16-cell stage, and (4) was limited to the endoderm of the trochophore. Granular bodies were found throughout the embryo. Mitochondria, found throughout the cytoplasm by first cleavage, were absent from the hyaloplasm of the unfertilized egg. The cytoplasm of the polar lobe was similar to that of the surrounding endoplasm. The localization of morphogenetic determinants was thus accompanied by cytoplasmic organelle localization in *Chaetopterus*. The results are interpreted in terms of localization mechanisms involving the embryo cortex and ground substances.

INTRODUCTION

Cleavage subdivides and segregates substances in the egg. This process is called localization, and localization patterns specify the differentiation of cells and tissues (see Wilson, 1928; Davidson, 1976; Whittaker, 1979; Freeman, 1979, for reviews). Localization of morphogenetic determinants may be reflected in the organization of visible cytoplasmic structure. The most clear-cut example of this occurs in *Styela* and related ascidians. In these animals, plasms visible by light (Conklin, 1905) or electron microscopy (Berg and Humphreys, 1960) are segregated into specific cell lineages.

The localization of morphogenetic determinants has been shown experimentally in spirallian eggs (e.g., Wilson, 1904; Novikoff, 1938, Clement, 1952), but attempts to associate particular cell lineages with cellular structure in these eggs usually have been fruitless. Most such attempts have centered on the structure of the polar lobe (see Dohmen and Verdonk, 1979, for review). The egg of the mollusc, *Bithynia*, has a very small polar lobe, containing a structure called the vegetal body, which is associated with the development of lobe-dependent structures (Dohmen and Verdonk, 1979). On the other hand, no structures are restricted exclusively to the

Received 3 December 1980, accepted 23 January 1981.
Abbreviations: MFSW, millipore-filtered seawater.

polar lobes of *Mytilus* (Reverberi and Mancuso, 1961; Humphreys, 1964), *Ilyanassa* (Crowell, 1964), or *Dentalium* (Reverberi, 1970; Dohmen and Verdonk, 1979). This is not surprising, since the larger cytoplasmic organelles can be displaced by centrifugation without affecting subsequent polar lobe formation and normal development (Lillie, 1909; Wilson, 1929, 1930; Whitaker and Morgan, 1930; Morgan, 1933, 1935; Clement, 1968). These results led the early investigators to propose that morphogenetic determinants are localized in the cytoplasmic "ground substances" (Lillie, 1906, 1909) or in the cortex (Morgan, 1933, 1935). These hypotheses have not been tested using the resolution of the electron microscope.

Lillie (1906) showed that cytoplasmic granules, detectable by differential staining, are localized in *Chaetopterus* eggs and embryos. In addition, if the eggs are briefly treated with excess KCl, they redistribute their cytoplasmic components and form cilia, a process called "differentiation without cleavage" (Lillie, 1902; Brachet, 1937). Because of these observations, the embryo of *Chaetopterus* should provide an excellent system for analyzing cytoplasmic organization by electron microscopy. This paper presents morphological evidence that cytoplasmic components, seen and identified by electron microscopy, are localized to different regions of the egg, and that this localization is maintained as the embryo cleaves and cells differentiate. It also presents evidence regarding the organization of both the cortex and the ground substance.

MATERIALS AND METHODS

Embryonic material

Mature specimens of *C. pergamentaceus* were obtained from the Marine Resources Division, Marine Biological Laboratory, Wood Hole, MA, and removed from their tubes. Sexes were separated and groups of individuals were kept in large fingerbowls with running sea water. Worms remained healthy under these conditions for at least 2 weeks. Females were used only after they had been kept separate from males for at least 2 days. Gametes were obtained and handled by standard procedures (Costello and Henley, 1971). Eggs were obtained by cutting off parapodia, teasing the eggs out, and filtering them through four layers of cheesecloth. Eggs were then washed at least three times with Millipore-filtered sea water (MFSW). Sperm, which oozed from cut parapodia into MFSW, were used to fertilize eggs as soon as the sperm became motile. In all experiments reported, >95% of inseminated eggs cleaved normally and developed into trochophore larvae. Embryos were cultured at 23°C in MFSW in Syracuse dishes and their development was monitored by phase-contrast microscopy.

Cytological techniques

Embryos were fixed either in 4% paraformaldehyde; 5% glutaraldehyde in MFSW (Karnovsky, 1965); or 0.75% glutaraldehyde, 5% formalin, 3% NaCl, and 4.5% sucrose in 0.1 *M* phosphate buffer (Fahrenbach, 1969). Morphology was similar with both fixatives. Aldehyde fixation was for 10–20 min at 23°C. Specimens were washed with MFSW 2 h to overnight, postfixed 30 min in 1% OsO_4 in MFSW, dehydrated rapidly through ethanol, and embedded in Spurr's (1969) low viscosity medium. Thick (1.0 μm) sections were cut using glass knives on a Porter-Blum MT2-B ultramicrotome and stained with toluidine blue. Thin sections were cut using a diamond knife and stained with uranyl acetate and lead citrate (Reynolds, 1963).

RESULTS

Unfertilized egg

C. pergamentaceus eggs are shed as primary oocytes and spontaneously undergo germinal vesicle breakdown. Unfertilized eggs examined in this study were at first meiotic metaphase. The surface of the egg possessed microvilli, which penetrated a 1 μm thick fibrillar vitelline layer (Fig. 1). A layer of cortical granules lay below the plasma membrane, but these were absent in the region of the meiotic spindle (Fig. 2). The spindle region had a spongy appearance due to numerous non-membranous vesicles, which often contained granular material (granular bodies). The spongy region also contained networks of microtubules and endoplasmic reticulum (Fig. 2) and was completely devoid of yolk, mitochondria, and lipid, except at its periphery. Granular bodies appeared to be arranged in rays by the spindle (Fig. 2, inset). At high magnification, they were composed of particles, 18–20 nm in diameter, and often were found in chains (Fig. 4). The rest of the cytoplasm contained a variety of organelles, apparently distributed haphazardly (Figs. 1, 3). Yolk platelets were interspersed with lipid, mitochondria, smooth endoplasmic reticulum, and granular bodies structurally similar to those in the spindle (Fig. 3). At high magnification, the yolk had a paracrystalline structure (Fig. 5).

Cleaving egg

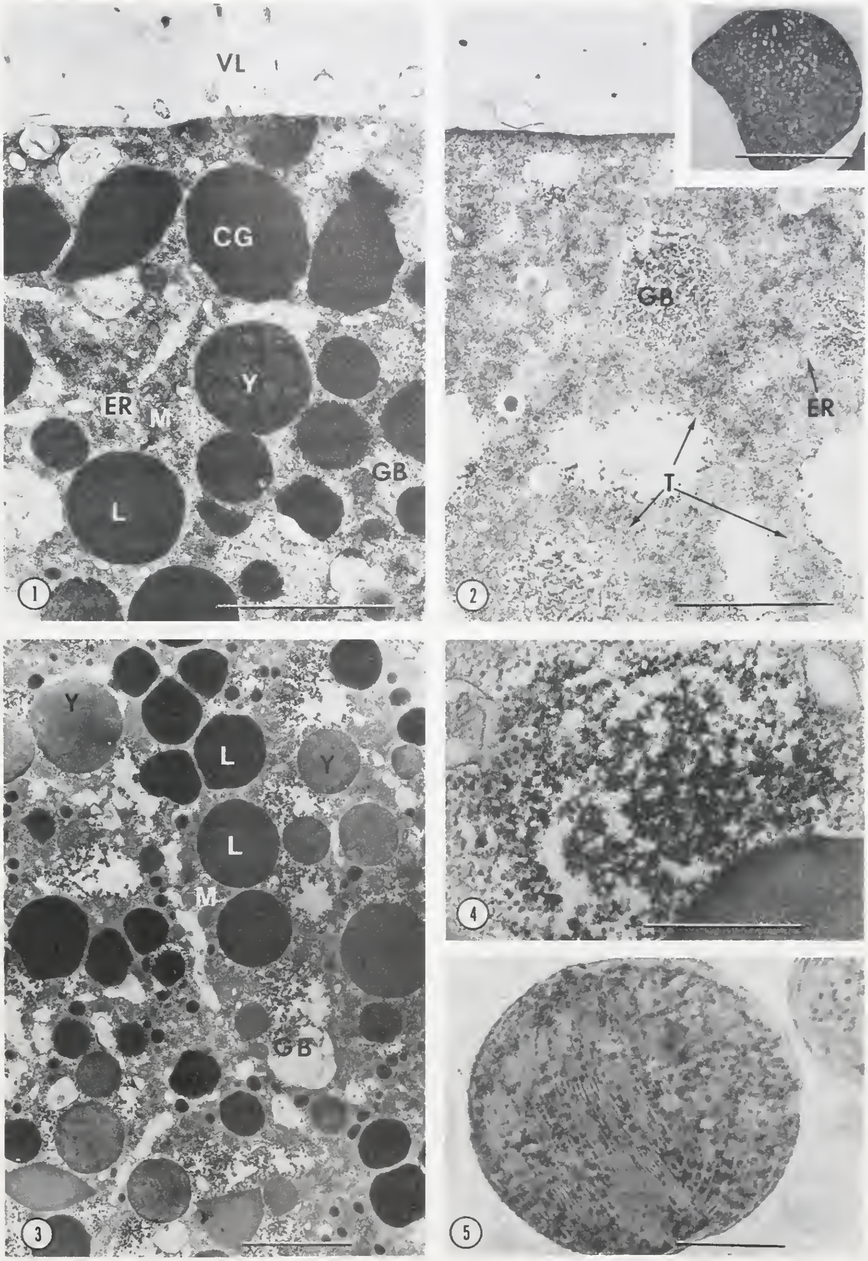
In the cleaving egg, the cortical region still possessed cortical granules. However, a 1 μm thick perivitelline space appeared between the vitelline layer and the plasma membrane (Fig. 6). Yolk, mitochondria, lipid, and smooth endoplasmic reticulum were distributed homogenously below the cortex. The spongy hyaloplasm became enclosed and contained occasional yolk platelets and lipid droplets, as well as numerous mitochondria in a matrix of microtubules and smooth endoplasmic reticulum (Fig. 7). The polar lobe showed no special structures. It contained the same cytoplasmic constituents as the surrounding cytoplasm, namely yolk, mitochondria, and lipid, which appeared to be arranged in rays running into the lobe (Fig. 8). The cortex of the polar lobe also appeared similar to that of the surrounding regions (Fig. 9). Granular bodies were interspersed with the cortical granules (Fig. 9). Cortical granules were reduced in number, but not completely absent, in cleavage constrictions. (Fig. 8).

Sixteen-cell embryo

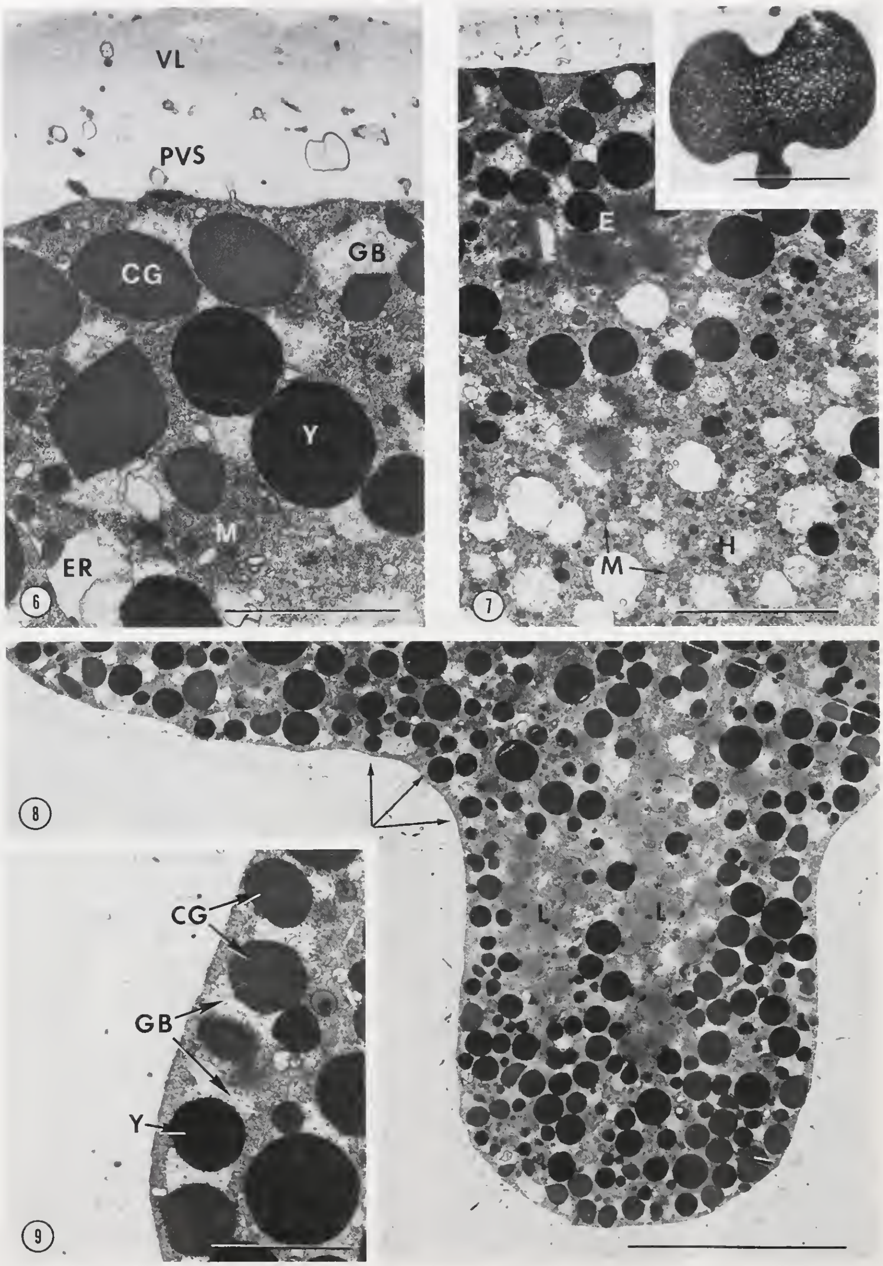
Figure 10 shows a blastomere of a 16-cell embryo. The cell was clearly polarized. The nucleus was eccentric, lying close to the cortex, which retained its cortical granules. The perinuclear hyaloplasm was spongy and possessed a network of microtubules and smooth endoplasmic reticulum (Fig. 11). Yolk and lipid were localized towards the segmentation cavity (Figs. 10, 12). Desmosomes anchored adjacent blastomeres to one another (Fig. 10, inset). Pores perforated the nuclear envelope.

Trochophore larva

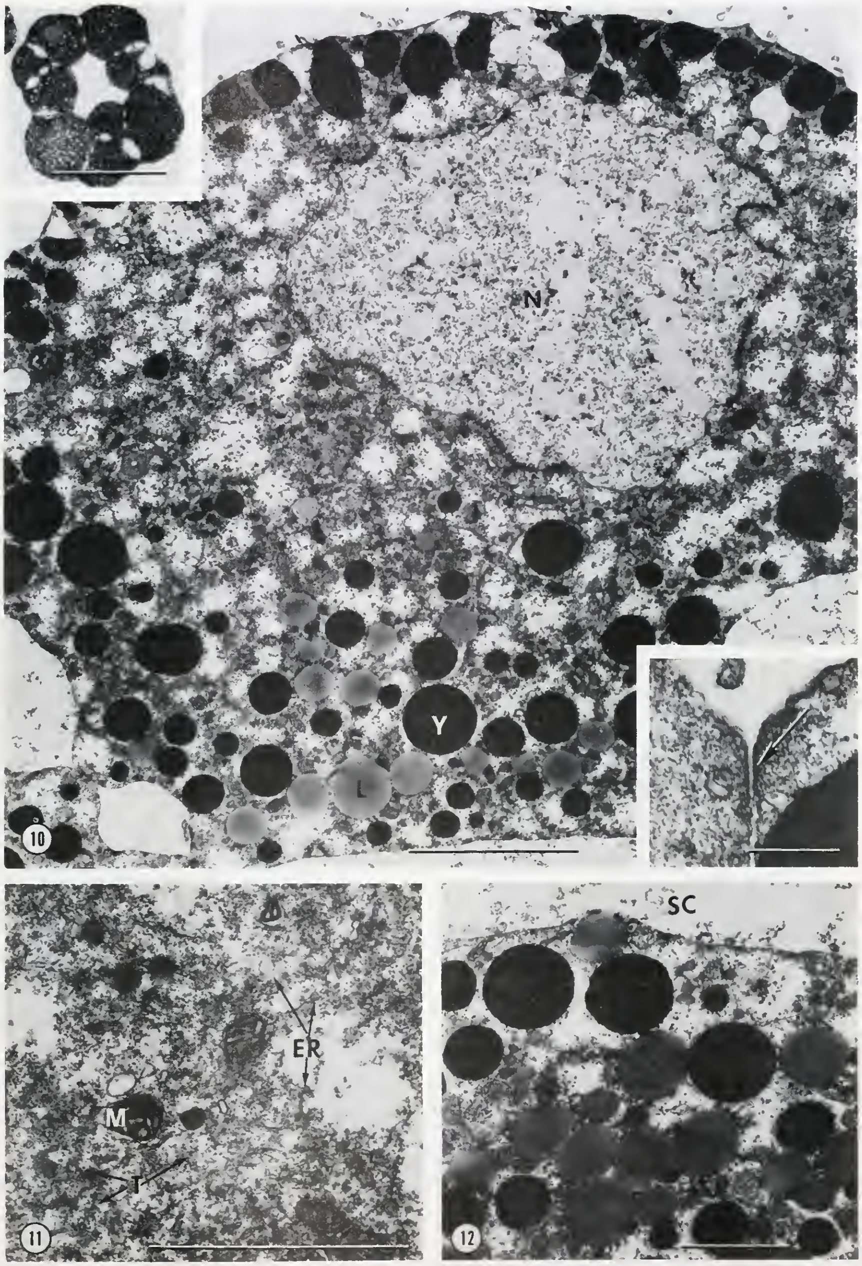
Embryos were followed to the early trochophore larval stage when the metatrochal band and apical organ were present, but the muscle bands had not fully



FIGURES 1–5. Figure 1. Section through the cortex of an unfertilized egg. Note cortical granules (CG), vitelline layer (VL), granular body (GB), mitochondrion (M), endoplasmic reticulum (ER), lipid (L), and yolk (Y). Scale bar = 2.0 μm. Figure 2. Section through the cortex of an unfertilized egg in the region of the spindle. Note granular body (GB), endoplasmic reticulum (ER), and microtubules (T), and the absence of other organelles. Scale bar = 2.0 μm. Inset: light micrograph of a thick section of an unfertilized egg, with meiotic spindle at the top. Note how hyaloplasm is arrayed by the spindle. Scale bar = 100 μm. Figure 3. Section through the endoplasm of an unfertilized egg showing granular bodies (GB), lipid (L), mitochondria (M), and yolk (Y). Scale bar = 2.0 μm. Figure 4. High magnification electron micrograph showing the fine structure of a granular body. Scale bar = 0.5 μm. Figure 5. High magnification electron micrograph showing the fine structure of a yolk granule. Scale bar = 0.5 μm.



FIGURES 6–9. Figure 6. Section through the cortex of a cleaving egg showing vitelline layer (VL), perivitelline space (PVS), cortical granules (CG), granular bodies (GB), endoplasmic reticulum, (ER), mitochondria (M) and yolk (Y). Scale bar = 2.0 μm. Figure 7. Low power electron micrograph of a section through a cleaving egg. Note the subcortical localization of the yolk and lipid of the endoplasm (E) and deeper localization of the hyaloplasm (H). Also note the presence of mitochondria (M) in the hyaloplasm. Scale bar = 5.0 μm. Inset: light micrograph of a cleaving egg. Scale bar = 100 μm. Figure 8. Low power electron micrograph through the polar lobe of a cleaving egg. Note rays of lipid (L) and similarity of polar lobe cytoplasm to surrounding endoplasm. Also note relative absence of cortical granules at furrows (arrows). Scale bar = 10 μm. Figure 9. Higher magnification electron micrograph through the cortex of the polar lobe showing association of granular bodies (GB) with cortical granules (CG) and yolk (Y). Scale bar = 2.0 μm.



FIGURES 10–12. Figure 10. Electron micrograph of a section through a blastomere of a 16-cell embryo. Note polarization of the cell with apical nucleus (N) and basal endoplasm with lipid (L) and yolk (Y). Scale bar = 5.0 μm. Upper inset: light micrograph of a section through a 16-cell embryo. Scale bar = 100 μm. Lower inset: electron micrograph showing a desmosome (arrow) at the junction between adjacent blastomeres. Scale bar = 1.0 μm. Figure 11. Section through the hyaloplasm of a 16-cell embryo. Note small traebeculae of endoplasmic reticulum (ER) and microtubules (T) and mitochondria (M). Scale bar = 2.0 μm. Figure 12. Section showing concentration of endoplasm near the segmentation cavity (SC). Scale bar = 2.0 μm.

differentiated and the archenteron lacked a well-defined lumen. Although the embryos were actively swimming, there was no evidence of "hatching" from the vitelline envelope. Instead, active cilia penetrated the envelope (Figs. 13, 14).

Cells near the apical end were columnar, with the nuclei away from the surface (Fig. 13). The cells had cilia and prominent cortical granules, mitochondria, and granular bodies.

Ectodermal cells of the metatroch (Fig. 14) had cilia with typical basal bodies, many mitochondria, a well-developed Golgi apparatus, vesicles of endoplasmic reticulum, and granular bodies. Cells were irregularly shaped and highly interdigitated. Desmosomes were at cell junctions near the embryo surface. Nuclear envelopes contained dense arrays of pores. Numerous microvilli lay among the cilia.

Figure 15 shows the postrochal region. Its cells had prominent microvilli but very few cilia. Endodermal cells containing yolk and lipid protruded between the ectodermal cells. All postrochal cells were irregularly shaped and very spongy, with abundant mitochondria and smooth endoplasmic reticulum.

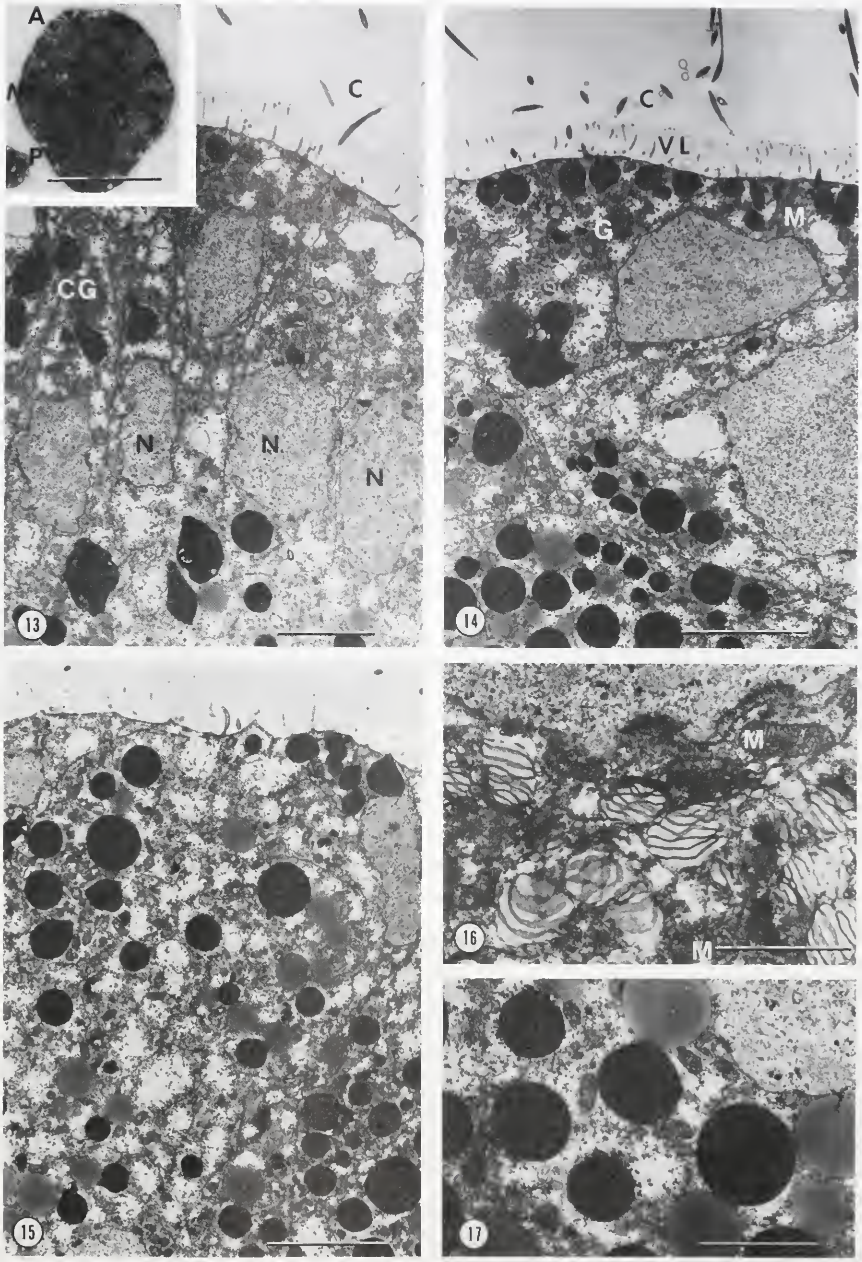
Mesodermal cells had numerous mitochondria, dilated vesicles of endoplasmic reticulum, and Golgi-like structures composed of stacks of membranes (Fig. 16).

Endodermal cells contained dense accumulations of yolk and lipid with associated mitochondria (Fig. 17) and fairly regularly-shaped nuclei with prominent nucleoli.

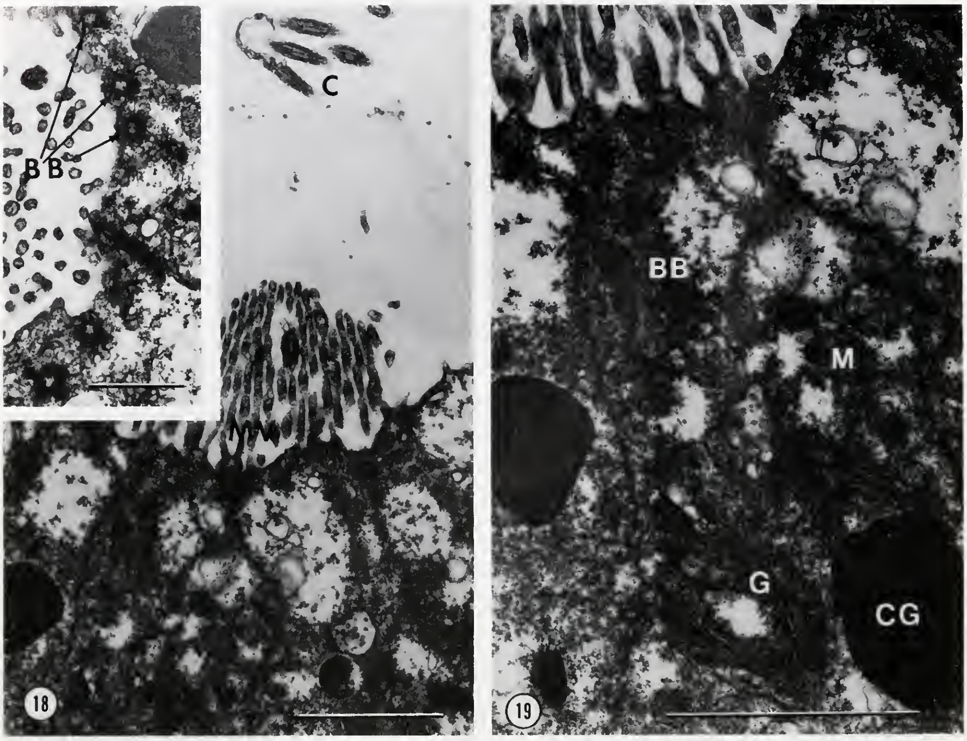
The apical organ was composed of several cilia surrounded by numerous microvilli up to 2 μm long, protruding from a group of cells forming a pit at the apical end (Fig. 13, inset; Fig. 18). Several cilia protruded from a single cell (Fig. 18, inset). Granular bodies were just below the surface, giving the apical organ a vesicular appearance (Figs. 18, 19). Mitochondria, Golgi apparatus, and vesicular endoplasmic reticulum were prominent. Cortical granules were present but deeper within the cells (Fig. 19).

Discussion

In an elegant series of studies, Lillie (1906, 1909) demonstrated the asymmetric localization of cytoplasmic components in *Chaetopterus* eggs and embryos, and the characteristic rearrangements of such components, which sequester them into different blastomeres. Based on staining properties, Lillie (1906) recognized (1) an ectoplasm, shown in the present study to be composed of cortical granules with interspersed granular bodies and vesicles of endoplasmic reticulum; (2) two granular endoplasms, shown here to be composed of yolk and lipid with interspersed mitochondria, granular bodies, and endoplasmic reticulum, and (3) a granule-free endoplasm, shown here to contain granular bodies in a matrix of microtubules and endoplasmic reticulum. For clarity, I call the granule-free endoplasm the hyaloplasm (*cf.* Wilson, 1928). Lillie (1909) recognized the morphogenetic importance of this material and noted that it is derived from the "residual substance" of the germinal vesicle. Its strong basophilia (Lillie, 1909) indicates the presence of acidic macromolecules. The present investigation showed the granular bodies to be composed of 18–20 nm structures aggregated into chains and larger granules. These small granules are approximately the size of ribosomes. The granular bodies are similar in structure to the polar granules of *Drosophila* (Mahowald, 1962) and the germinal granules of amphibians (*e.g.*, Williams and Smith, 1971). Which components of the granules of insects and amphibians carry morphogenetic significance is not known. These latter granules are associated with germinal differentiation, whereas the granular bodies in *Chaetopterus* were found in all tissues of the trochophore and in all parts of the early embryo.



FIGURES 13–17. Figure 13. Electron micrograph showing the apical region of a trochophore larva. Note columnar shape of cells, cilia (C), cortical granules (CG), and nuclei (N). Also note absence of yolk and lipid from ectoderm. Scale bar = 5.0 μm. Inset: light micrograph of a section through a trochophore larva to show locations of sections shown in Figures 13–15. A = apical region; M = metatrochal band; P = postrochal region. Scale bar = 100 μm. Figure 14. Electron micrograph of a section through the metatrochal band. Note cilia (C) penetrating the vitelline layer (VL). Also note Golgi apparatus (G) and mitochondria (M). Scale bar = 5.0 μm. Figure 15. Electron micrograph of a section showing the postrochal region. Note that endodermal cells containing yolk and lipid approach the surface but are still surrounded by ectodermal cells. Also note relative lack of cilia. Scale bar = 5.0 μm. Figure 16. Section through a mesodermal cell. Note dilated stacks of membranes and numerous mitochondria (M). Scale bar = 2.0 μm. Figure 17. Typical section through an endodermal cell. Scale bar = 2.0 μm.



FIGURES 18–19. Figure 18. Section through the apical organ showing microvilli (MV) and cilia (C). Inset: three basal bodies may be seen in one cell of the apical organ (BB, arrows). Scale bar = 1.0 μm. Figure 19. Higher magnification electron micrograph of apical organ cells showing basal bodies (BB), cortical granules (CG), and Golgi apparatus (G). Scale bar = 2.0 μm.

In the unfertilized egg, the hyaloplasm was in the spindle region and excluded structures such as mitochondria, yolk, lipid, and cortical granules (ectoplasmic defect of Lillie, 1906). After fertilization, mitochondria moved into the area in abundance, although it still contained little yolk or lipid.

The structure of the hyaloplasm persisted throughout cleavage and gastrulation, although its position in the embryo changed. In accordance with previous reports (Lillie, 1902, 1906, 1909), the hyaloplasm was at the interior of the cleaving egg and eventually moved to more superficial regions. In the trochophore, the hyaloplasm was mostly in the ectoderm.

The only change in the egg cortex upon fertilization was the appearance of a perivitelline space. This was not due to cortical granule breakdown, because the cortical granules persisted to the trochophore. Humphreys (1967) reported a similar situation in *Mytilus*. The endoplasm was not distinct from the ectoplasm during early development.

At first cleavage, the endoplasm was peripheral and completely enclosed the hyaloplasm. Endoplasm filled the polar lobe. This contradicts Lillie's (1906) report that the polar lobe is primarily ectoplasmic. Whitaker and Morgan (1930) also found the polar lobe to be endoplasmic. Mead (1895) reported astral rays penetrating the polar lobe. The lipid aggregates shown in this report may have been those rays.

With the exception of Dohmen and Verdonk (1979), using *Bithynia*, no specialized structures of potential morphogenetic importance have been detected in studies of molluscan polar lobes (Reverberi and Mancuso, 1961; Crowell, 1964; Humphreys, 1964; Reverberi, 1970) although some structures have been reported to be concentrated in polar lobes (*e.g.*, mitochondria, Reverberi, 1970; double-membrane vesicles, Crowell, 1964). Since *Bithynia* has a small polar lobe, Dohmen and Verdonk (1979) suggested that small polar lobes might generally contain special structures. The annelid, *Chaetopterus*, also has a small polar lobe, but the present study showed no lobe-specific structures.

By the 16-cell stage, the endoplasm was oriented toward the basal end of all cells, as reported by Lillie (1906). This arrangement is maintained through cleavage and may be responsible for sequestering the endoplasm into the presumptive endodermal cells into which it ultimately passes. In the trochophore larva, endoplasmic structures were lacking in ectodermal cells, while cortical granules were lacking in endodermal cells as reported by Lillie (1906). This is consistent with the polarization of cells in the 16-cell embryo, although the endodermal cell lineage is not completely segregated at that stage (Mead, 1897).

Lillie (1906) reported that the apical organ consists of a flagellum protruding from cells lacking ectoplasm. The present report showed that the flagellum was a bundle of cilia and that the cells of the apical organ did contain cortical granules, although the granules were not positioned superficially.

Certain features of cytoplasmic localization were striking in *Chaetopterus* development. Ooplasmic segregation resulted in larval cells with distinct and characteristic cellular inclusions. The mechanism underlying this localization is not yet known with certainty. Microtubules and endoplasmic reticulum were arranged in a network throughout the cytoplasm. Since this network appeared to surround at least some of the localized organelles, it may contribute to cytoplasmic localization. Lillie (1906, 1909) and Wilson (1928) stressed the importance of such "ground substances" in localization.

Centrifugal forces sufficient to stratify the large organelles did not result in abnormal development of *Chaetopterus* (Lillie, 1906, 1909; Wilson, 1929, 1930) or other spirallians (Morgan, 1933, 1935; Clement, 1968). Electron microscopic examinations of centrifuged spirallian eggs have not reported any persisting microtubule or endoplasmic reticulum network, but neither have these been previously reported in uncentrifuged eggs. Vesicles of endoplasmic reticulum are found throughout centrifuged *Mytilus* eggs, however (Humphreys, 1962). Clearly an analysis of centrifuged *Chaetopterus* eggs by electron microscopy is warranted.

It has been proposed that morphogenetic information is localized in the cortex of the egg (e.g., Wilson, 1928; Davidson, 1976), but cellular structures that might localize morphogenetic determinants have not been detected. If such information is localized in the form of informational RNA molecules, as has often been supposed (see Davidson, 1976, for review), granular bodies, if they are shown to contain informational RNA, would be prime candidates for such localization. Because of their organization within the egg, they are probably sequestered along with other cytoplasmic components in *Chaetopterus* embryos.

ACKNOWLEDGMENTS

I thank Dr. Y. H. Kang for his assistance and cooperation and Dr. W. A. Anderson for a critical reading of the manuscript. This research was supported by NIH grant RR-08016.

LITERATURE CITED

BERG, W. E., AND W. J. HUMPHREYS. 1960. Electron microscopy of four-cell stages of the ascidians *Ciona* and *Styela. Develop. Biol.* **2:** 42–60.

BRACHET, J. 1937. La différenciation sans clivage dans l'oeuf de Chaetoptère envisagé aux points de vue cytologique et métabolique. *Arch. Biol.* **48:** 561–589.

CLEMENT, A. C. 1952. Experimental studies on germinal localization in *Ilyanassa.* I. The role of the polar lobe in determination of the cleavage pattern and its influence in later development. *J. Exp. Zool.* **121:** 593–625.

CLEMENT, A. C. 1968. Development of the vegetal half of the *Ilyanassa* egg after removal of most of the yolk by centrifugal force compared with the development of animal halves of similar composition. *Develop. Biol.* **17:** 165–186.

CONKLIN, E. G. 1905. The organization and cell-lineage of the ascidian egg. *J. Acad. Nat. Sci. Phil.* **13:** 1–119.

COSTELLO, D. P., AND C. HENLEY. 1971. Pp. 63–66 in *Methods for obtaining and handling marine eggs and embryos.* Second edition. Marine Biological Laboratory, Woods Hole, MA.

CROWELL, J. 1964. The fine structure of the polar lobe of *Ilyanassa obsoleta. Acta. Embryol. Morphol. Exp.* **7:** 225–234.

DAVIDSON, E. H. 1976. *Gene activity in early development.* Second edition. Academic Press, New York. 452 pp.

DOHMEN, M. R., AND N. H. VERDONK. 1979. The ultrastructure and role of the polar lobe in development of molluscs. Pp. 3–27 in S. Subtelny and I. R. Konigsberg, Eds., *Determinants of spatial organization.* Academic Press, New York.

FAHRENBACH, W. H. 1969. The morphology of the eye of *Limulus.* II. Ommatidia of the compound eye. *Z. Zellforsch.* **93:** 451–483.

FREEMAN, G. 1979. The multiple roles which cleavage can play in the localization of developmental potential. Pp. 53–76 in S. Subtelny, and I. R. Konigsberg, Eds., *Determinants of spatial organization.* Academic Press, New York.

HUMPHREYS, W. J. 1962. Electron microscope studies on eggs of *Mytilus edulis. J. Ultrastruct. Res.* **7:** 467–487.

HUMPHREYS, W. J. 1964. Electron microscope studies of the fertilized egg and two-cell stages of *Mytilus edulis. J. Ultrastruct. Res.* **10:** 244–262.

HUMPHREYS, W. J. 1967. The fine structure of cortical granules in eggs and gastrulae of *Mytilus edulis. J. Ultrastruct. Res.* **17:** 314–326.

KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27:** 137a–138a.

LILLIE, F. R. 1902. Differentiation without cleavage in the egg of the annelid *Chaetopterus pergamentaceus. Wilhelm Roux' Arch. Develop. Biol.* **14:** 477–499.

LILLIE, F. R. 1906. Observations and experiments concerning the elementary phenomena of embryonic development in *Chaetopterus. J. Exp. Zool.* **3:** 153–268.

LILLIE, F. R. 1909. Polarity and bilaterality of the annelid egg. Experiments with centrigual force. *Biol. Bull.* **16:** 54–79.

MAHOWALD, A. P. 1962. Fine structure of pole cells and polar granules in *Drosophila melanogaster. J. Exp. Zool.* **151:** 201–215.

MEAD, A. D. 1895. Some observations on maturation and fecundation in *Chaetopterus pergamentaceus* Cuvier. *J. Morphol.* **10:** 313–317.

MEAD, A. D. 1897. The early development of marine annelids. *J. Morphol.* **13:** 227–326.

MORGAN, T. H. 1933. The formation of the antipolar lobe in *Ilyanassa. J. Exp. Zool.* **64:** 433–467.

MORGAN, T. H. 1935. The separation of the egg of *Ilyanassa* into two parts by centrifuging. *Biol. Bull.* **68:** 280–295.

NOVIKOFF, A. B. 1938. Embryonic determination in the annelid, *Sabellaria vulgaris.* II. Transplantation of polar lobes and blastomeres as a test of their inducing capacities. *Biol. Bull.* **74:** 211–234.

REVERBERI, G. 1970. The ultrastructure of *Dentalium* egg at the trefoil stage. *Acta Embryol. Exp.* **1970:** 31–43.

REVERBERI, G., AND V. MANCUSO. 1961. The constituents of the egg of *Mytilus* as seen at the electron microscope. *Acta Embryol. Morphol. Exp.* **4:** 102–121.

REYNOLDS, E. W. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17:** 208–212.

SPURR, A. R. 1969. A low-viscosity epoxy embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26:** 31–43.

WHITAKER, D., AND T. H. MORGAN. 1930. The cleavage of polar and antipolar halves of the egg of *Chaetopterus. Biol. Bull.* **58:** 145–149.

WHITTAKER, J. R. 1979. Cytoplasmic determinants of tissue differentiation in the ascidian egg. Pp. 29–51 in S. Subtelny and I. R. Konigsberg, Eds., *Determinants of spatial organization.* Academic Press, New York.

WILLIAMS, M. A., AND L. D. SMITH, 1971. Ultrastructure of the "germinal plasm" during maturation and early cleavage in *Rana pipiens. Develop. Biol.* **25:** 568–580.

WILSON, E. B. 1904. Experimental studies on germinal localization. I. The germ regions in the egg of *Dentalium. J. Exp. Zool.* **1:** 1–72.

WILSON, E. B. 1928. *The cell in development and heredity.* Third edition, with corrections. MacMillan, New York. 1232 pp.

WILSON, E. B. 1929. The development of egg-fragments in annelids. *Wilhelm Roux' Arch. Develop. Biol.* **117:** 180–210.

WILSON, E. B. 1930. Notes on the development of fragments of the fertilized *Chaetopterus* egg. *Biol. Bull.* **59:** 71–80.

Reference: *Biol. Bull.*, **160**: 240–249. (April, 1981)

THE LYMPHOMYELOID (HEMOPOIETIC) SYSTEM OF THE ATLANTIC NURSE SHARK, *GINGLYMOSTOMA CIRRATUM*

RAGNAR FÄNGE AND ARTUR MATTISSON

Department of Zoophysiology, University of Göteborg, Sweden

ABSTRACT

The lymphomyeloid system of nearly adult nurse sharks, *Ginglymostoma cirratum,* was investigated. Lymphomyeloid structures detectable by the naked eye at dissection are the epigonal organ and the spleen. Microscopic examination shows that the epigonal organ produces granulocytes and lymphocytes. The white pulp of the spleen is lymphoid, whereas the red pulp is mainly erythropoietic. Cells with the morphological characteristics of plasma cells occur in the epigonal organ and the spleen. Peroxidase-positive granulated cells are found in the epigonal organ. In contrast to many other elasmobranchs, the nurse shark lacks the Leydig organ, *i.e.* the lymphomyeloid structure of the esophagus; but the epigonal organ is well developed and averages 0.60% of the body weight. The spleen weighs about 0.26% of the body weight. The mode of life of the nurse shark in shallow tropical waters probably puts a considerable demand on its immune system. Cells produced by the epigonal organ may be important in immune responses and in inflammatory processes.

INTRODUCTION

Elasmobranch fish lack bone marrow and lymph nodes, but possess a thymus gland (at least in early life), a spleen, and voluminous lymphomyeloid tissues associated with the gonads, esophagus, or both. Diffuse lymphoid infiltrations may occur in the intestine or elsewhere. The gonad-associated lymphomyeloid tissue, often called the epigonal organ, reaches considerable size in certain sharks, such as the basking shark, *Cetorhinus maximus* (Matthews, 1950). Elasmobranch species lacking epigonal organs possess a similar tissue, termed the Leydig organ, in the esophagus (Fänge, 1977). The epigonal and Leydig organs, both relatively little known structures, resemble bone marrow and lymph nodes of higher vertebrates. Most previous studies of elasmobranch lymphomyeloid tissues concern species from temperate or cool waters. The present work deals with the lymphomyeloid system of the nurse shark, *Ginglymostoma cirratum* (family Orectolobidae), that lives in tropical and subtropical parts of the Atlantic Ocean.

MATERIALS AND METHODS

Six not-fully-adult specimens of *Ginglymostoma cirratum* trapped off Puerto Rico were kept in running seawater for several weeks and fed minced molluscs. The nurse shark is one of the few sharks that can be maintained in captivity for long periods (Clark, 1963). Five sharks were killed and perfused with formalin solution. The sixth animal was investigated immediately after killing, without fixation. The main lymphomyeloid structures were isolated by dissection and weighed. Histological sections were stained with eosin-haematoxylin. Due to the paucity of

Received 14 October 1981, accepted 22 January 1981.

Abbreviation: MGG, May-Grünwald-Giemsa.

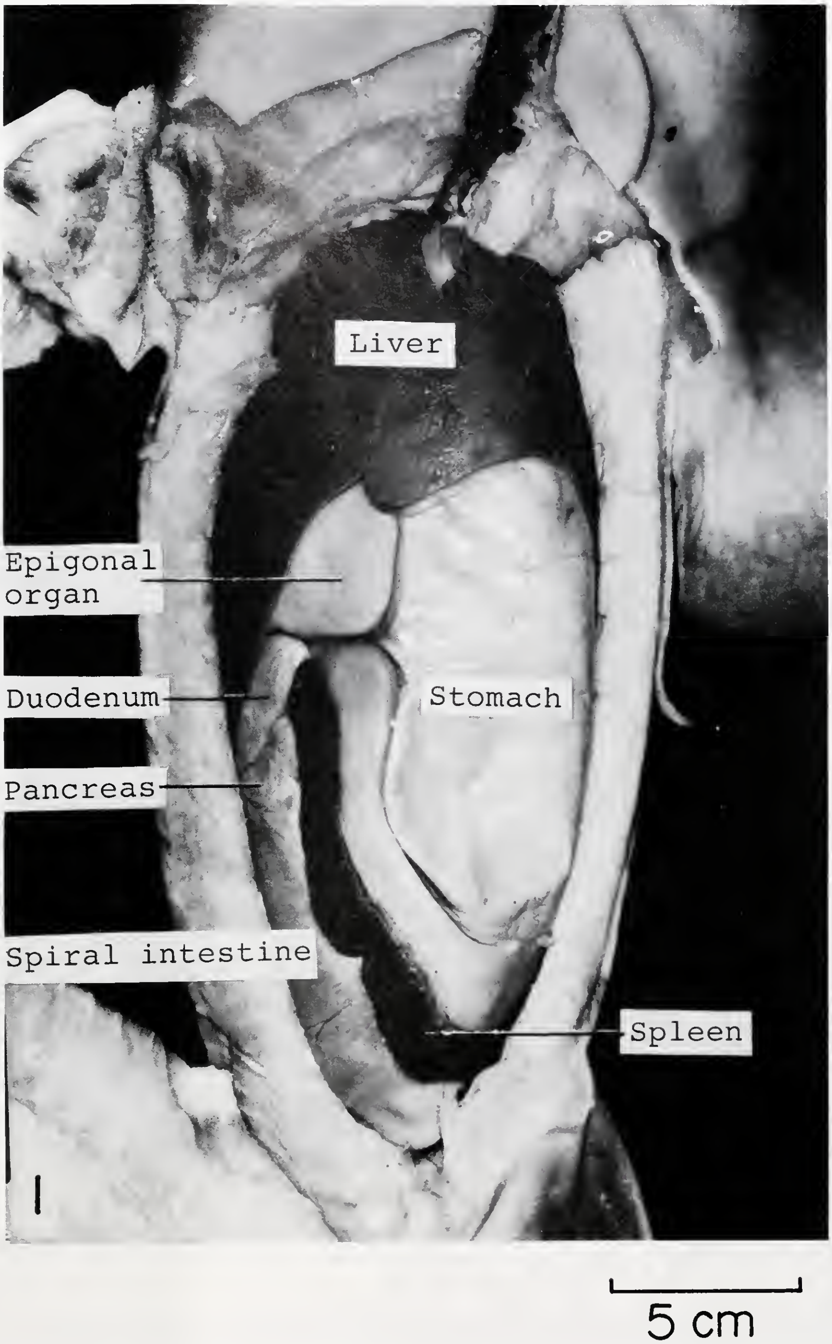
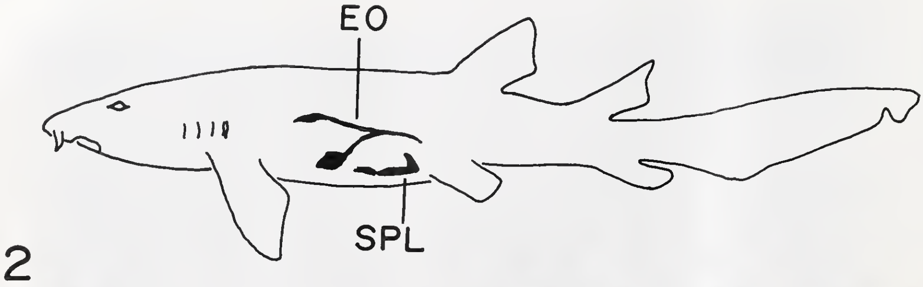
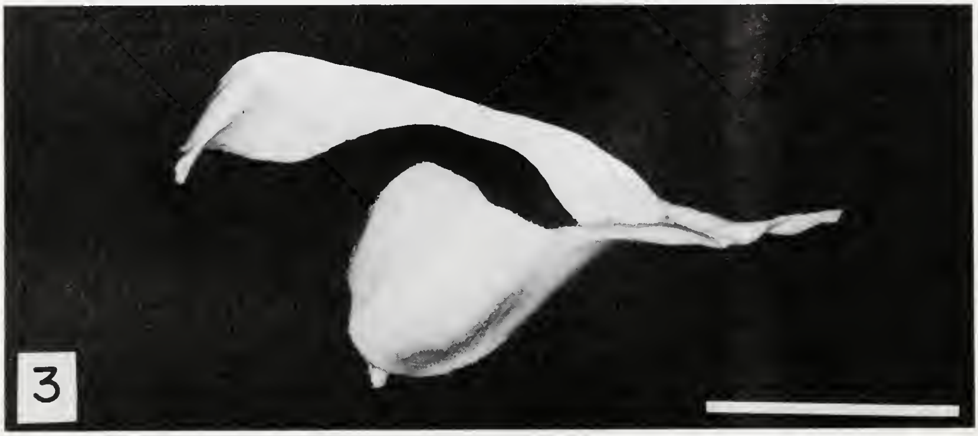

Liver
Epigonal organ
Duodenum
Stomach
Pancreas
Spiral intestine
Spleen
5 cm

FIGURE 1. Ventral view of abdominal viscera of a female nurse shark after perfusion. The club-shaped right anterior part of the epigonal organ is in the center of the figure.





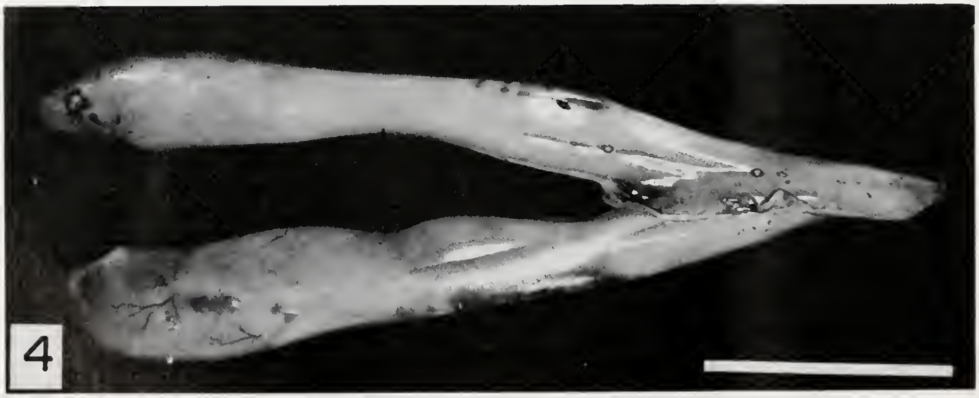


FIGURE 2. Diagram of the positions in the body of the epigonal organ (EO) and the spleen (SPL) of a female.

FIGURE 3. Ventral view of formalin-fixed female epigonal organ. The club-shaped right anterior lobe (low center) contains the single ovary. This is embedded in lymphomyeloid tissue and not visible. Photograph with background blacked out. Bar = 5 cm.

FIGURE 4. Ventral view of unfixed freshly dissected male epigonal organ. Testicular tissue is visible in the right anterior lobe (low left). Photograph with background blacked out. Bar = 5 cm.

fresh material, formalin-fixed material also was used for electron microscopy, after post-fixation in 1% OsO_4 dissolved in 0.1 *M* sodium cacodylate buffer, pH 7.3; and embedding in Epon 812. The sections were stained with uranyl acetate and lead citrate and examined with a Hitachi HS-8 instrument.

TABLE I

Weights of epigonal organ and spleen in relation to body weight (b.w.) of the nurse shark, Ginglymostoma cirratum.

No.	Sex	Body weight (g)	Epigonal organ (% b.w.)	Spleen (% b.w.)
1	Female	1930	0.79	0.21
2	Female	2311	0.43	0.22
3	Female	2750	0.62	0.29
4	Male	2897	0.79	0.36
5	Female	3800	0.42	0.26
6	Female	6700	0.57	0.24
Mean		3398	0.60	0.26

Cytochemical tests for peroxidase activity were performed on frozen sections of formalin-fixed material using the method of De Olmos and Heimer (1977).

Blood smears and imprints from the freshly investigated specimen were air-dried, fixed 15 min in methanol, and stained with May-Grünwald-Giemsa (MGG).

RESULTS

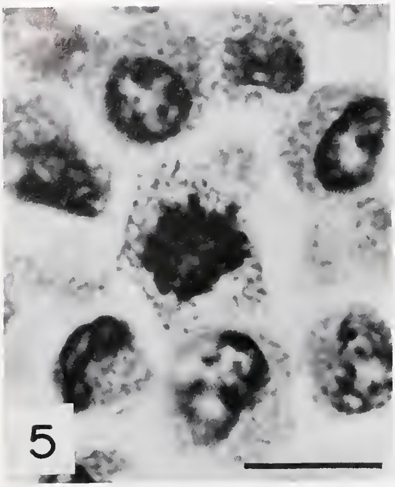
The epigonal organ and the spleen were the only lymphomyeloid structures examined that are detectable by the naked eye during dissection (Fig. 1, 2). Neither the thymus nor the Leydig organ are visible macroscopically, but a thymus undoubtedly exists during larval stages, as has been described from other species of elasmobranchs.

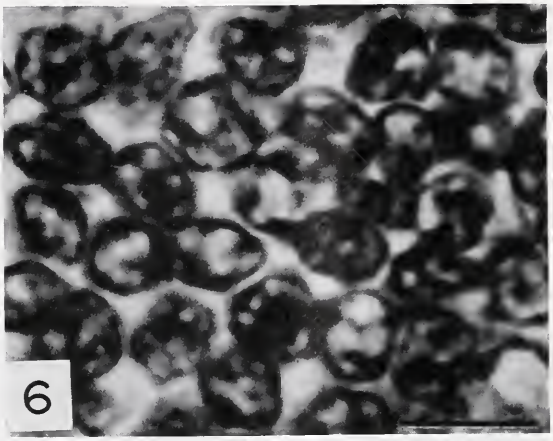
Epigonal organ

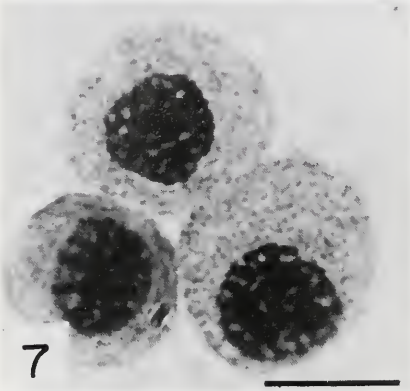
The epigonal organ, a whitish Y-shaped structure in the abdomen (Fig. 3, 4), averages 0.60% of the shark's body weight (Table I). In the male the two testes, each about the same size as the other, are embedded within the left and right anterior parts of the epigonal organ (Fig. 4). In the female the right anterior part of the epigonal organ is enlarged and club-like and contains the right ovary (Fig. 3). The left ovary is rudimentary or absent. From its dorsal attachment the right anterior part of the female epigonal organ spirals, or curves, towards the ventral side of the body cavity, forming a prominent tissue lobe between the liver and the duodenal-splenic-pyloric complex (Fig. 1). The left anterior part of the female epigonal organ is thin, elongate, and devoid of gonadal tissue. In both sexes the epigonal tissues extend caudally to the rectal gland. The sexually immature fishes examined had gonads weighing 5–10% of the corresponding epigonal organs.

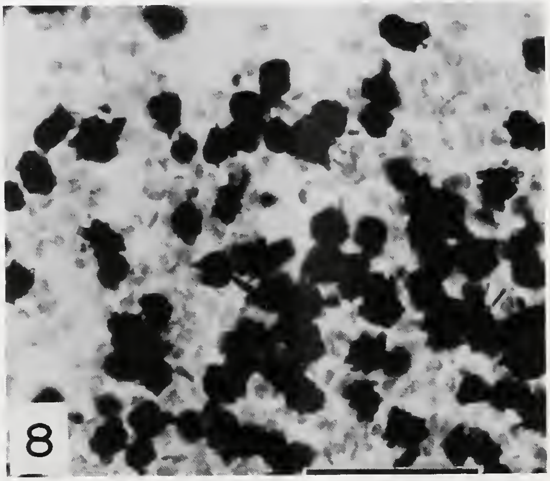
A peritoneum covers the epigonal organ. The parenchyma consists of large amounts of leucocytes, in various stages of development, in the meshes of a stroma formed by connective tissue and the walls of blood vessels and blood lacunes. Granulocytes with a round, oval, or slightly segmented nucleus (Fig. 5, 7) are the most common types of cells. The slight nuclear segmentation indicates that the granulocytes of the epigonal organ are less mature than those of the circulating blood, which usually have 2-, 3- or 4-lobed nuclei (Fig. 9). Granulocytes often undergo mitosis, probably in the promyelocyte stage (Fig. 5). Numerous cells, seemingly granulocytes, contain peroxidase-positive cytoplasmic granules (Fig. 8).

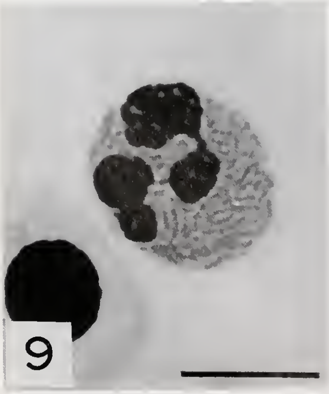
In imprint preparations the leucocytic granules appear oval, rod-shaped, or round, and are weakly eosinophilic. MGG-stained imprints show, in addition to granulocytes, numerous non-granulated cells with basophilic cytoplasm and large

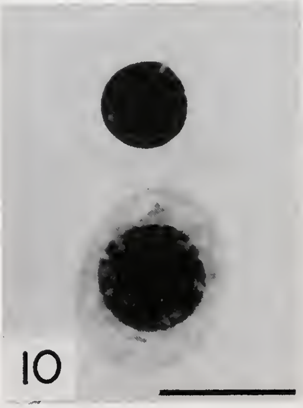












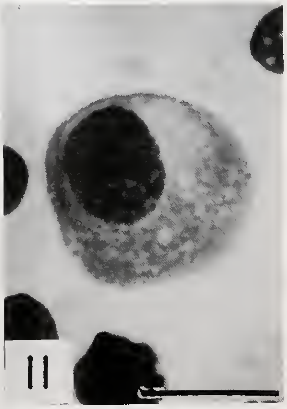


FIGURE 5. Histological section of the epigonal organ stained with eosin-haematoxylin, showing promyelocytes. One cell in mitosis. Bar = 10 μm.

FIGURE 6. Histological section of the epigonal organ stained with eosin-haematoxylin, showing follicle-like aggregation of lymphocytes. Bar = 10 μm.

nuclei. Many of these cells are granuloblasts or lymphoblasts. Other non-granulated cells, difficult to distinguish from the blast-type cells, fulfill morphological criteria for plasma cells: an eccentric nucleus with aggregations of chromatin and a strongly basophilic cytoplasm with a pale area next to the nucleus (centrosphere and Golgi apparatus). Nuclei with little or no cytoplasm around them are small lymphocytes, which are very abundant scattered among the granulocytes or in follicle-like aggregations (Fig. 6).

In the electron microscope, one or a few crystalloid rhomboid inclusions usually were seen in the granules of the granulocytes (Fig. 12).

Spleen

The spleen is of normal appearance for an elasmobranch. It is smaller than the epigonal organ, about 0.26% of the body weight (Table I). It consists of red and white pulp. White pulp (periarterial sheaths of Malpighian bodies) consists of rounded masses of densely packed lymphocytes. The diameters of the lymphoid masses vary between about 200 μm and 1200 μm. The lymphoid masses contain no granulocytes or erythrocytes; but blast-type cells, probably lymphoblasts, are scattered among large numbers of small lymphocytes. A few small arteries and capillaries penetrate the lymphoid masses. The red pulp contains cells of the erythrocytic line mixed with nongranulated cells and a few granulocytes. Ellipsoids, *i.e.* concentric lamellae of connective tissue around arterioles, are present. Blast cells, probably erythroblasts, and cells with the morphological characteristics of plasma cells are observed in imprint preparations (Fig. 11). Immature red cells with large round nuclei are found in the red pulp of the spleen and in the circulating blood (Fig. 10).

Under electron microscopy granules of spleen granulocytes appear ovoid and contain fibrous inclusions (Fig. 13). Plasma cells are very abundant (Fig. 14).

DISCUSSION

The epigonal organ is the most prominent lymphomyeloid structure in nearly adult specimens of the nurse shark, *Ginglymostoma cirratum*. Curiously enough, Gohar and Mazhar (1964) observed no epigonal organ in an adult female Red Sea nurse shark, *Nebrius concolor*, a species closely related to *Ginglymostoma cirratum*. However, lymphomyeloid organs are dynamic structures that change size and cell composition in relation to endocrine factors (Yoffey and Courtice, 1970). The epigonal organ may have a tendency to involute during maturing of the gonads, although it is not likely to disappear completely.

The main function of the epigonal organ, as indicated by its cell composition, is production and storage of leucocytes, primarily granulocytes. However, it also contains lymphocytes and probably great numbers of plasma cells, although the latter were identified by light microscopy only.

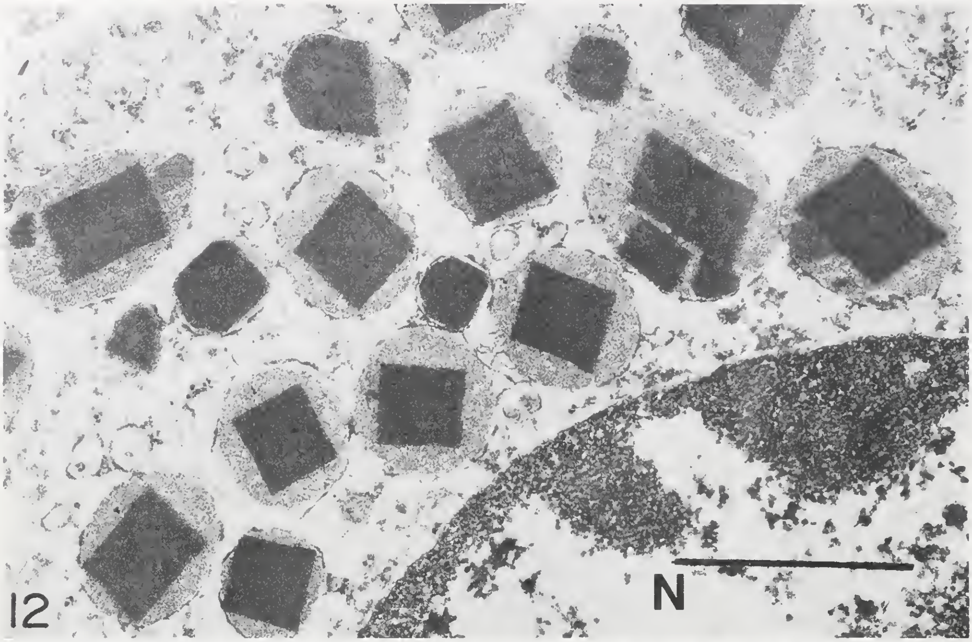
FIGURE 7. MGG-stained imprint of epigonal organ, showing immature granulocytes (promyelocytes). Bar = 10 μm.

FIGURE 8. Peroxidase-containing cells in frozen section of epigonal organ. Bar = 50 μm.

FIGURE 9. MGG-stained blood smear, containing granulocyte with segmented nucleus. Bar = 10 μm.

FIGURE 10. MGG-stained blood smear, showing one mature and one immature erythrocyte. Bar = 10 μm.

FIGURE 11. MGG-stained imprint of the spleen, showing plasma cell. Bar = 10 μm.



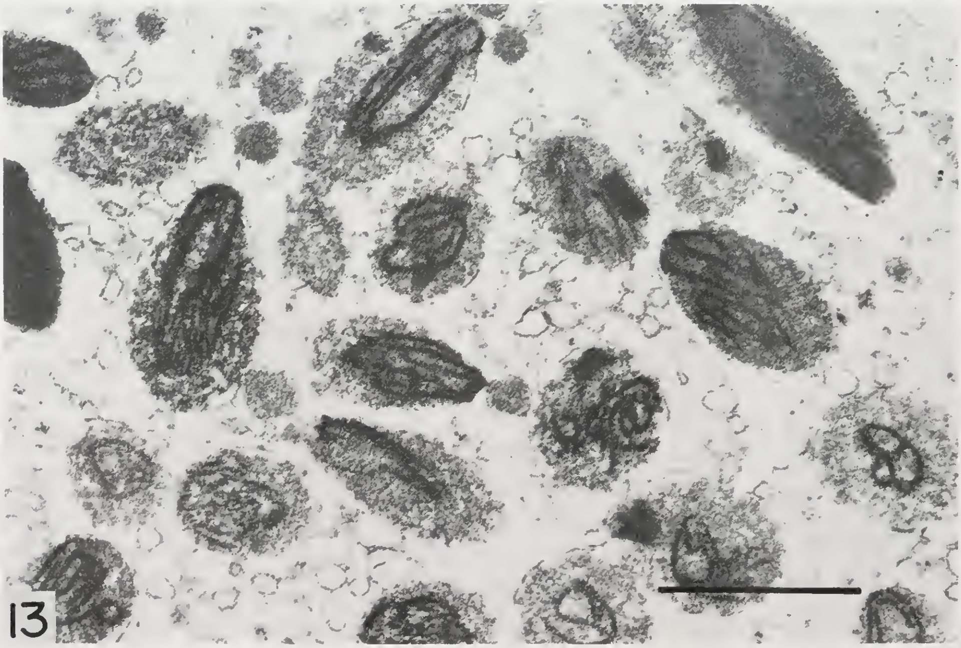


FIGURE 12. Electron micrograph of epigonal organ; part of granulocyte showing granules with rhomboid crystalloid inclusions. Bar = 1 μm. N = nucleus.

FIGURE 13. Electron micrograph of spleen. Granulocyte cytoplasm showing granules with fibrous inclusions. Bar = μm.

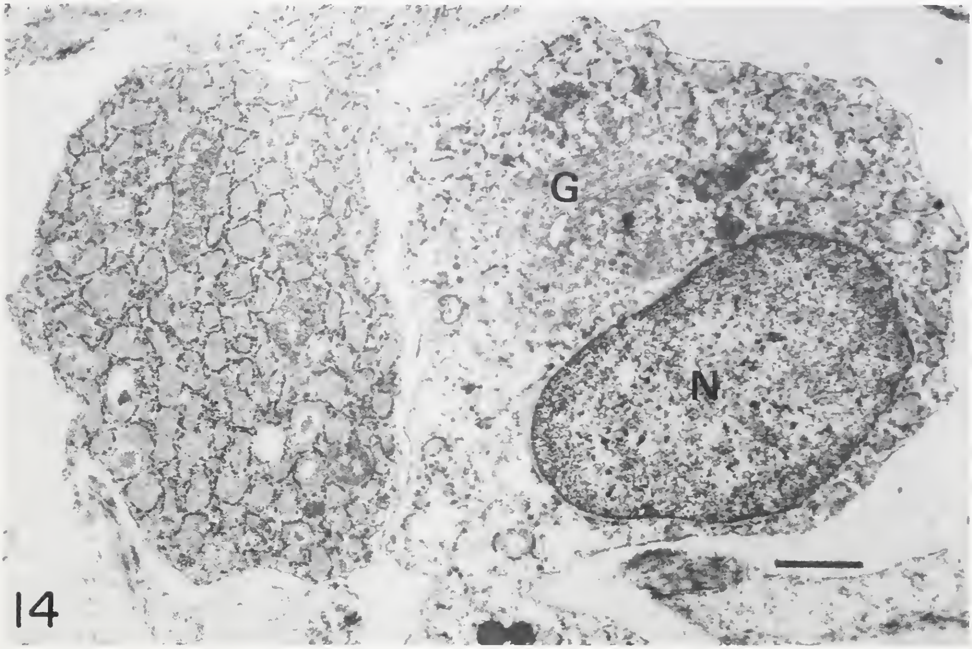


FIGURE 14. Electron micrograph of a plasma cell in the spleen. Bar = 1 μm. G = Golgi apparatus; N = nucleus.

Leucocytes are known to contain hydrolytic lysosomal enzymes. In accordance with this, the epigonal organs of *Ginglymostoma* and other elasmobranchs are rich in glycosidases (Fänge *et al.*, 1980). The finding in the epigonal organ of cells containing peroxidase contrasts with a result by Fey (1966), who found little peroxidase in blood leucocytes of the shark, *Scyliorhinus canicula*. The divergence in results could be a species difference. Leucocytic peroxidases are assumed to take part in bacteria killing systems (*cf.* Selvaraj *et al.*, 1980).

The nurse shark lives close to the bottom in shallow tropical and subtropical inshore waters. In these surroundings, liability to infections and mechanical damage may be greater than in the open sea, necessitating efficient immune and wound-healing mechanisms. Immunological studies have shown that the nurse shark produces two types of immunoglobulins and has a complement system, and that in the adult stage about 50% of its plasma proteins are immunoglobulins (Fidler *et al.*, 1969; Rudikoff *et al.*, 1970; Ross and Jensen, 1973). The blood contains cells similar to mammalian T lymphocytes, and cells cytotoxic towards a variety of target cells (Lopez *et al.*, 1974; McKinney *et al.*, 1977). The nurse shark has a highly differentiated immune system, and efficient wound healing (Reif, 1978). The well developed epigonal organ may supplement the spleen in antibody production, and leucocytes originating in the epigonal organ may function in inflammatory processes during wound healing. In histological structure the epigonal organ rather closely resembles hemopoietic bone marrow, although it produces only white cells. The initial stage of erythropoiesis takes place in the red pulp of the spleen.

Thus Zapata (1980) found ultrastructural evidence of splenic erythropoiesis in rays (*Raja, Torpedo*). However, red cells may mature in the peripheral blood (Kanesada, 1956).

Of the about 600 living species of elasmobranchs (Nelson, 1976), only a few have been examined for appearance and distribution of lymphomyeloid tissues. Studies from our laboratory (Fänge, 1977, and unpublished observations) show an epigonal organ but no Leydig organ in *Ginglymostoma cirratum* (present work), *Rhinoptera bonasus, Heterodontus francisci,* or *Negaprion brevirostris.* Both epigonal and Leydig organs occur in *Raja* species, *Scyliorhinus canicula, Scyliorhinus stellaris,* and *Sequalus acanthias.* Elasmobranchs that possess a Leydig organ but no epigonal organ or only traces of one are *Somniosus microcephalus, Etmopterus spinax,* and *Torpedo* species. In no elasmobranchs are both the epigonal and Leydig organs missing. Hologephalians (chimaeroid cartilaginous fish) lack both epigonal organs and Leydig organs, but have similar lymphomyeloid tissues in cavities of the cartilage skeleton (Stahl, 1967).

ACKNOWLEDGMENTS

Animals were maintained at the Laboratory of Neurobiology, San Juan, Puerto Rico. Thanks are due to the Director, Dr. Jose El Castillo, his staff, and Dr. Sven Ebbeson, for good working facilities. I thank Dr. P. Leuken for advice on histochemistry and Ms. Lolyn Lopez for expert technical assistance. This work was supported by the Swedish National Science Research Council.

LITERATURE CITED

CLARK, E. 1963. The maintenance of sharks in captivity, with a report in their instrumental conditioning. Pp. 115–149 in P. W. Gilberts, Ed., *Sharks and Survival.* D. C. Heath and Co., Boston.

DE OLMOS, J., AND L. HEIMER. 1977. Mapping of collateral projections with the horseradish peroxidase method. *Neurosci. Lett.* **6:** 107–114.

FÄNGE, R. 1977. Size relations of lymphomyeloid organs in some cartilaginous fish. *Acta Zool. (Stockholm)* **58:** 125–128.

FÄNGE, R., G. LUNDBLAD, K. SLETTENGREN, AND J. LIND. 1980. Glycosidases in lymphomyeloid (hematopoietic) tissues of elasmobranch fishes. *Comp. Biochem. Physiol.* **67B:** 527–532.

FEY, F. 1966. Vergleichende Hämozytologie niederer Vertebraten. III. Granulozyten. *Folia Haematol.* **86:** 1–20.

FIDLER, J. E., L. W. CLEM, AND P. A. SMALL, JR. 1969. Immunoglobulin synthesis in neonatal nurse shark (*Ginglymostoma cirratum*). *Comp. Biochem. Physiol.* **31:** 365–371.

GOHAR, H. A. F., AND F. M. MAZHAR. 1964. Internal anatomy of selachians from the northwestern Red Sea. *Publications of the Marine Biological Station Al-Ghardaqa (Egypt)* **13:** 145–240.

KANESADA, A. 1956. A phylogenetic survey of hemocytopoietic tissues in submammalian vertebrates. *Bull. Yamaguchi Med. Sch.* **4:** 1–21.

LOPEZ, D. M., M. M. SIGEL, AND J. C. CLEM. 1974. Phylogenetic studies on T cells. 1. Lymphocytes of the shark with differential response to phytohemagglutinin and concanavalin A. *Cell. Immunol.* **10:** 287–293.

MACKINNEY, E. C., C. L. PETTEY, D. M. LOPEZ, AND M. M. SIGEL. 1977. Cytotoxicity of nurse shark (*Ginglymostoma cirratum*) leucocytes. Pp. 217–224 in A. E. Ellis, J. B. Solomon, and J. D. Hoston, Eds., *Developmental Immunology.* Elsevier, New York.

MATTHEWS, L. H. 1950. Reproduction in the basking shark, *Cetorhinus maximus* (Gunner). *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* **234:** 247–299.

NELSON, J. S. 1976. *Fishes of the World.* John Wiley and Sons, New York. 1416 pp.

Reif, W.-E. 1978. Wound healing in sharks. Form and arrangement of repair scales. *Zoomorphologie* **90:** 101–111.

Ross, G. D., and J. A. Jensen. 1973. The first component (C in) of the complement system of the nurse shark (*Ginglymostoma cirratum*). I. Hemolytic characteristics of partially purified C in. *J. Immunol.* **110:** 175–182.

Rudikoff, S., E. W. Voss, and M. M. Sigel. 1970. Biological and chemical properties of natural antibodies in the nurse shark, *Ginglymostoma cirratum. J. Immunol.* **105:** 1344–1352.

Selvaraj, R. J., J. M. Zgliczynski, B. B. Paul, and A. J. Sbarra. 1980. Chlorination of reduced nicotinamide adenine nucleotide by myeloperoxidase. A novel bactericidal mechanism. *J. Reticuloendothelial Soc.* **27:** 31–38.

Stahl, B. J. 1967. Morphology and relationships of the Holocephali with special reference to the venous sytsem. *Bull. Mus. Comp. Zool.* **135:** 141–213.

Yoffey, J. M., and F. C. Courtice. 1970. *Lymphatics, Lymph and the Lymphomyeloid Complex.* Academic Press, New York. 942 pp.

Zapata, A. 1980. Splenic erythropoiesis and thrombopoiesis in elasmobranchs. An ultrastructural study. *Acta Zool. (Stockholm)* **61:** 59–64.

Reference: *Biol. Bull.*, **160:** 250–264. (April, 1981)

COMPOSITION OF THE BLOOD SERUM OF DEEP-SEA FISHES

ROBERT W. GRIFFITH

Department of Biology, Southeastern Massachusetts University,
North Dartmouth, Massachusetts 02747

ABSTRACT

Serum osmolarity, chloride, urea, protein, and trimethylamine oxide were measured in 15 shallow water marine teleosts, 6 elasmobranchs, 9 deep benthic teleosts, and 24 midwater teleosts. Amino acids, carbohydrates, phosphate, and hematocrits were determined for some species from these four groups. Elasmobranchs had high osmolarity (1035 mOsm/l) because of high serum urea (363 m*M*/l), TMAO (66 m*M*/l), and chloride (295 m*M*/l). Shallow water teleosts had low osmolarity (444 mOsm/l), chloride (176 m*M*/l), urea (4 m*M*/l), and TMAO (14 m*M*/l). Deep benthic teleosts had higher osmolarities and chloride levels (576 mOsm/l, 242 m*M*/l) than shallow water teleosts, as did midwater teleosts (561 mOsm/l, 267 m*M*/l). Serum TMAO was high in benthic (51 m*M*/l), but not midwater (12 m*M*/l) teleosts, and urea was low in midwater (1.0 m*M*/l) and benthic (1.5 m*M*/l) groups. Stress and morbidity raise osmolarity and chloride in marine teleosts and may account for high values in midwater and benthic fishes, which were sampled after considerable trauma. The data suggest that deep-sea teleosts osmoregulate as do shallow water species, and do not support the notion that osmoregulatory specializations, such as ureosmotic regulation, evolve more rapidly in the deep sea. Very low serum proteins (0.8 g/100 ml) and hematocrits (<10%) in midwater teleosts possibly relate to buoyancy or low metabolism.

INTRODUCTION

Marine fishes regulate their blood sera in three basic ways. Hagfish blood is isosmotic and, for most ions, isoionic to sea water. Blood osmolarities of sharks, their kin, and the coelacanth are similar to that of their environment, but their ion concentrations are lower because they maintain high levels of organic solutes such as urea and trimethylamine oxide (ureosmotic regulation). In the third pattern, found in marine teleosts and lampreys, blood serum is both hyposmotic and hypoionic to sea water (hyposmotic regulation).

The hagfishes' osmoregulation may not have changed from that of the earliest vertebrate ancestor, but the other two mechanisms depart radically from the condition in marine invertebrates, probably because these groups passed through a freshwater or estuarine evolutionary stage (Pang *et al.*, 1977). A number of reasons could explain why teleosts never developed ureosmotic regulation when they reinvaded the sea, even though this type of regulation probably evolved independently in the chondrichthians, the coelacanth, and the crab-eating frog, *Rana cancrivora* (Griffith and Pang, 1979). Among these possible reasons are: (1) They did not develop tolerance to elevated tissue urea levels. (2) Early teleost ancestors that entered the sea did not have enzymes of ureogenesis or had them in too low titers. (3) The teleosts evolved more recently than the chondrichthians and coelacanths,

---

Received 7 November 1980, accepted 2 February 1981.

Abbreviations: WHOI, Woods Hole Oceanographic Institution; TMAO, trimethyl amine oxide.

and so did not have time to develop urea retention. (4) Ureosmotic regulation is only advantageous in a constant marine environment, and most teleosts whose blood chemistry has been studied inhabit inshore or estuarine areas during some stage in their life histories. (5) Ureosmotic regulation is energetically feasible only in large fishes, such as chondrichthians and *Latimeria*, which protect their young against passive urea loss during development. (6) Once the earliest marine teleosts developed hyposmotic regulation, changing to another osmoregulatory strategy was impossible because of evolutionary channelization.

Some of these hypotheses are testable. Despite a contrary report (Brown and Cohen, 1960), a variety of teleosts have all enzymes of the ornithine-urea cycles (Huggins *et al.*, 1969) and some have them in higher titers than certain urea-retaining chondrichthians (Read, 1967, 1971b). Although urea is moderately toxic to teleosts (Rasmussen and Rasmussen, 1977; Griffith *et al.*, 1979), it probably is not toxic enough at levels found in elasmobranch tissues to seriously impede evolution of ureosmotic regulation. Although the four remaining hypotheses are not subject to direct experimental proof, hypotheses 3 (insufficient time) and 4 (estuarine existence of most teleosts) can be tested indirectly by studying the body fluid chemistry of deep-sea teleosts.

Deep-sea fishes include many of the most ancient teleost lineages, providing a long evolutionary history in the sea, and they live in constant salinities with no exposure to a dilute environment in which urea retention would be a disadvantage. Because ureosmotic regulation in elasmobranchs uses less energy than hyposmotic regulation in teleosts (Griffith and Pang, 1979), urea retention should be of great advantage to teleosts in the deep sea, an energy-deprived environment where conservation of energy would have selective advantage.

Ureosmotic regulation is not the only possible osmoregulatory specialization deep-sea teleosts might have evolved to conserve energy. They could retain bulk quantities of organic solutes other than urea (e.g., trimethylamine oxide) to elevate blood osmolarity without raising blood electrolytes to toxic levels. Alternatively, they could build up ion (NaCl) concentrations to a condition like that in hagfishes, which are isosmotic and isoionic to sea water. Blaxter *et al.* (1971) suggested that, in contrast, midwater fishes might have lower blood solutes than other teleosts to provide buoyancy without using energy.

Few studies have dealt with osmoregulation in deep-sea fishes. Blaxter *et al.* (1971), testing whether midwater fishes use dilute body fluids for buoyancy, investigated blood osmolarity of several such fishes. Data also exist on the blood and tissue electrolytes of certain deep benthic fishes (Whitt and Prosser, 1971; Prosser *et al.*, 1975; Fänge *et al.*, 1972). However, no study has examined whether deep-sea fishes have evolved osmoregulatory specializations, such as urea retention, for coping with the constant high salinity and low available energy characteristic of their environment. This report seeks such specializations in a broad array of midwater and benthic deep-sea fishes.

Materials and Methods

All specimens were collected September 1974 to August 1975. The near-shore and surface oceanic fishes listed in Table I were collected in the vicinity of Woods Hole, Massachusetts (*Brevoortia, Opsanus, Gadus, Morone, Pomatomus, Stenotomus, Cynoscion, Scomber, Makaira, Palinurichthyes,* and *Paralichthyes*), near Bermuda (*Sphryaena*), or in the open ocean between Bermuda and Woods Hole (*Carynx, Cheilopogon,* and *Coryphaena*). Most were caught on hook and line, but

TABLE I

Blood serum constituents of some inshore marine and surface oceanic teleost fishes. (N = number of specimens tested for osmolarity, chloride, urea, protein and osmotic deficit, with TMAO usually measured in fewer. Condition: A = all specimens alive, D = all dead. Values are mean ± SE when N ⩾ 3, and mean alone when N < 3)

Species	N	Condition	Osmolarity (mOsm/l)	Chloride (m*M*/l)	Urea (m*M*/l)	Protein (g/100 ml)	TMAO (m*M*/l)	Osmotic deficit (m*M*/l)
Brevoortia tyrannus	2	A	408	181	1.7	3.4	8.0	36.3
Opsanus tau	10	A	333 ± 3	151 ± 5	3.4 ± 0.4	2.4 ± 0.2	4.8	22.8
Gadus morhua	1	A	416	162	2.3	5.7	6.9	82.8
Cheilopogon exsiliens	2	A	578	234	1.9	2.9	—	108.1
Morone saxatilus	6	A	424 ± 3	139 ± 7	10.4 ± 2.8	6.9 ± 0.5	19.2 ± 5.1	116.4
Pomatomus saltatrix	14	A	436 ± 4	159 ± 3	4.8 ± 0.8	6.6 ± 0.4	31.8 ± 11.0	81.4
Carynx chrysos	1	A	486	241	1.3	3.4	1.3	1.4
Coryphaena hippurus	8	A	464 ± 22	183 ± 13	4.3 ± 1.3	4.8 ± 0.5	14.5 ± 7.4	79.2
Stenotomus chrysops	12	A	370 ± 3	177 ± 2	2.0 ± 0.3	3.2 ± 0.2	37.4	−23.4
Cynoscion regalis	1	A	425	150	3.8	5.4	30.6	90.6
Sphyraena borealis	3	A	394 ± 9	144 ± 9	2.4 ± 0.7	6.7 ± 0.1	7.5	96.1
Scomber scomber	1	A	487	171	7.4	5.7	9.1	128.5
Makaira albidans	2	D	587	168	3.7	8.3	12.8	234.5
Palinurichthyes perciformis	1	A	492	208	9.0	5.3	5.8	61.2
Paralichthyes dentatus	3	A	363 ± 11	172 ± 15	2.1 ± 0.3	4.5 ± 0.4	10.6	6.8

Gadus and *Makaira* were collected using set long lines, *Cheilopogon* was caught in neuston nets, and *Opsanus* specimens were obtained from the Marine Biological Laboratory's specimen company. The elasmobranchs listed in Table II were col-

TABLE II

Blood chemistry of some marine elasmobranchs. (N = number of specimens tested for all factors except TMAO (for which fewer were generally tested). Condition: A = all specimens alive, D = all dead. Values are mean ± SE when N ⩾ 3 and mean alone when N < 3).

Species	N	Condition	Osmolarity (mOsm/l)	Chloride (m*M*/l)	Urea (m*M*/l)	Protein (g/100 ml)	TMAO (m*M*/l)	Osmotic deficit (m*M*/l)
Raja erinacea	3	A	991 ± 23	268 ± 13	416 ± 19	3.9 ± 0.4	41.4	2.4
Mustelus canis	2	A	1004	272	406	3.1	90.0	−36.0
Squalus acanthias	2	A	1005	275	368	3.2	22.0	65.0
Carcharhinus obscurus	1	A	1092	278	386	2.3	76.0	74
Prionace glauca	9	A	1036 ± 24	256 ± 4	391 ± 9	1.7 ± 0.2	99.0 ± 6	34
Centroscymnus coelolepis	1	D	1079	423	208	0.5	66.0	−41

lected using hook and line in the vicinity of Woods Hole (*Mustelus, Squalus,* and *Raja*), by set line on the continental shelf off the coast of Massachusets (*Prionace* and *Carcharhinus*), or by bottom trawl on the continental slope off New England (*Centroscymnus*). The deep benthic fishes listed in Table III were collected with otter or beam trawls in the Nares Abyss south of Bermuda at depths of *ca.* 5000 m (*Bathypterois*, a brotulid, and a rattail), on the continental slope off Massachusetts at depths of *ca.* 500 m (*Lepophidium, Macrozoarces, Urophycis,* and *Nematonurus*), or in Hudson's Canyon (*Nezumia* and *Antimora*). All the specimens reported as midwater fishes (Table IV) were collected using Issac-Kidd midwater trawls towed obliquely from 1000 m to the surface for about 2 h, mostly in the Sargasso Sea (many in, or near, cold core Gulf Stream rings; see Jahn, 1976, for details).

Identification of many of the species was problematical, particularly among the midwater and deep benthic groups, because workable keys were not available at sea and specimens could not be preserved. Expert identification of many of the midwater fishes was provided at sea by Drs. Andrew Jahn and James Craddock of Woods Hole Oceanographic Institution (WHOI). Species not positively identified are listed separately at the end of Table IV as "unidentified A", etc. Among the benthic fishes, *Nezumia bairdi* and *Antimora rostrata* were identified positively by Dr. Richard Haedrich (WHOI). The continental slope species (*Nematonurus, Urophycis, Lepophidium,* and *Macrozoarces*) and the abyssal *Bathypterois* were only tentatively identified. The unidentified abyssal rattail and brotulid are the same specimens reported on by Harvey and Steinhauer (1976). Fishes not positively identified were included in this study because their omission would impair generalizations that could be made about the blood chemistry of deep-sea fishes.

Blood of relatively large inshore teleosts and elasmobranchs was collected by puncturing the caudal artery with a large-gauge needle and withdrawing the blood into a heparinized syringe. The hearts of smaller specimens or moribund midwater

TABLE III

*Blood serum chemistry of some marine teleosts from the Continental Slope, Hudson's Canyon and abyss. (N = number of specimens tested for osmolarity, chloride, and osmotic deficit, with fewer often tested for urea, protein, and TMAO. Condition: D = all specimens dead at sampling. Values are means ± SE when N ⩾ 3, mean alone when N < 3. * = identification uncertain.)*

Group/species	N	Condition	Osmolarity (mOsm/l)	Chloride (m*M*/l)	Urea (m*M*/l)	Protein (g/100 ml)	TMAO (m*M*/l)	Osmotic deficit (m*M*/l)
Abyssal fishes								
Bathypterois sp.*	2	D	775	319	0.9	—	—	136
Rattail*	1	D	617	274	1.2	2.6	6.3	62
Brotulid*	1	D	591	236	0.7	—	—	118
Hudson's Canyon								
Nezumia bairdi	3	D	486 ± 41	227 ± 13	1.5 ± 0.1	2.0	—	31
Antimora rostrata	1	D	485	171	1.2	2.0	97	45
Continental slope								
*Nematonurus armatus**	8	D	567 ± 22	263 ± 15	1.3 ± 0.2	2.9 ± 0.4	49 ± 10	−9
*Urophycis tenuis**	3	D	608 ± 40	292 ± 22	1.1 ± 0.2	2.5	—	23
*Lepophidium cervinum**	1	D	538	203	4.0	—	—	128
*Macrozoarces americanus**	1	D	520	202	—	—	—	116

TABLE IV

Blood serum chemistry of some midwater marine teleosts. (N = number of specimens tested for osmolarity, chloride, and osmotic deficit, with fewer often measured for urea, protein, and TMAO. Condition: A = all specimens alive, D = all dead, AD = some alive and some dead. Values are mean ± SE when $N \geqslant 3$, and mean alone when $N < 3$).

Species	N	Condition	Osmolarity (mOsm/l)	Chloride (m*M*/l)	Urea (m*M*/l)	Protein (g/100 ml)	TMAO (m*M*/l)	Osmotic deficit (m*M*/l)
Derichthys serpentinus	2	D	753	331	2.0	1.6	—	89
Nemichthys scolopaceus	4	D	706 ± 39	352 ± 23	0.9 ± 0.3	0.5	0.4	1
Bathylagus bericoides	1	A	374	155	0.1	—	—	64
Gonastoma elongatum	8	A	433 ± 14	204 ± 8	1.1 ± 0.2	0.5 ± 0.2	4.4 ± 1.0	20
Gonastoma elongatum	20	D	638 ± 30	313 ± 17	1.5 ± 0.0	0.5 ± 0.0	10.1	0
Argyropelycus aculeatus	10	D	578 ± 33	272 ± 19	1.3 ± 0.1	1.6 ± 0.1	22.8	10
Malocosteus niger	1	D	607	304	0.7	0.3	—	−2
Chauliodus sloani	8	AD	621 ± 73	306 ± 35	0.7 ± 0.2	0.5 ± 0.1	21.2 ± 9.7	−13
Stomias boa	2	D	427	217	—	—	—	−7
Echiostoma barbatum	2	D	463	184	—	—	35.0	60
Photonectes margarita	1	D	363	197	1.0	—	—	−32
Diaphus rafinesque	6	AD	550 ± 14	247 ± 13	1.5 ± 0.2	1.6 ± 0.2	14.7	40
Lampadena speculigera	1	D	462	198	0.8	—	—	65
Hygophum heigenmani	1	D	527	195	1.5	—	—	136
Ceratias holbrooki	1	A	545	275	0.8	0.4	6.2	−12
Anoplogaster cornuta	1	A	408	189	0.8	1.9	0.0	29
Scopelogadus beani	9	A	489 ± 33	239 ± 19	0.6 ± 0.2	0.4 ± 0.1	4.6 ± 0.5	6
Gempylus serpens	2	D	708	334	1.9	1.5	22.2	16
Nesiarchus nasutus	2	D	652	337	2.3	—	—	−24
Unidentified A	1	D	656	321	0.5	0.7	6.0	8
Unidentified B	2	D	668	336	0.8	0.3	16.8	−22
Unidentified C	2	D	604	298	1.2	0.4	19.2	−3
Unidentified D	1	D	608	312	0.9	0.4	6.6	−24
Unidentified E	1	A	522	256	0.7	0.4	3.3	6
Unidentified F	1	D	657	311	0.4	0.4	—	35

or benthic fishes were punctured and the blood collected directly in non-heparinized capillary tubes. Blood was refrigerated and later centrifuged to separate plasma or serum from red cells. The serum or plasma was then frozen at −20°C for subsequent analyses. Serum and plasma are both referred to as serum, since the only difference expected between the two in the factors studied was slightly lower protein in serum. Hematocrits were routinely taken for most specimens.

Blood serum was analyzed using minor modifications of the microtechniques described in detail by Pickford *et al.* (1969). Serum osmolarity was determined on a Mechrolab (now Hewlett-Packard) vapor-pressure osmometer equipped with Hamilton microliter syringes which made it possible to analyze volumes of *ca.* 15 μl. Serum chloride was measured using an Aminco-Cotlove chloride titrator with sample volumes of 5 μl. Serum urea was estimated on 10 μl samples using the Hycel diacetyl monoxime method, with absorbance read on a Zeiss M4 QIII-PM QII spectrophotometer. Serum protein levels were determined spectrophotometrically by the method of Lowry *et al.* (1951). Trimethylamine oxide concentrations were estimated titrimetrically, in Conway diffusion dishes, by a modification of the technique of Forster *et al.* (1958). The reducing agent was metallic zinc, and the liberated free amine was absorbed in a boric acid buffer containing Bromcresol green indicator, and was titrated to a colorimetric end point with dilute HCl. Serum amino acids, carbohydrates, and phosphate were measured where the serum volume was sufficient. Amino acids were measured colorimetrically by the method of Troll and Cannon (1953); total carbohydrates by Anthrone reaction (Pickford *et al.*, 1969); and phosphate by the microtechnique of Lowry *et al.* (1953).

The contribution of unmeasured solutes to blood osmolarity can be approximated by adding the molar concentrations of two times chloride (*i.e.* assuming an equal number of monovalent cations to balance the chloride, and complete dissociation) to the other measured osmotically-active solutes (such as urea and trimethylamine oxide), and subtracting this sum from the measured osmolarity. These values of osmotic deficit do not include proteins, because of their uncertain osmotic contribution; nor do they include amino acids, carbohydrates, and phosphate, because of the limited number of species analyzed.

Because of the trauma to deep-sea fishes from net collection, most midwater and deep benthic specimens were in poor physiological condition and many were dead when sampled (Tables I–IV). Heartbeat was used to determine whether specimens were alive (A) or dead (D).

RESULTS

The sampling problem

Comparing results from living and dead specimens of *Gonastoma elongatum* shows which serum constituents change after death and how much (Table IV). Dead *Gonastoma* specimens had significantly higher osmolarity (47%) and chloride (53%). Serum urea and protein did not change significantly. This relationship seemed to apply to most midwater teleosts; dead specimens generally had higher osmolarity and chloride, but urea and protein did not differ consistently. Dead midwater fishes generally had more trimethylamine oxide (TMAO) than those alive when sampled. The possible effects of pressure changes on a membrane's permeability to solutes and solvents has been explored by Gordon (1970) and MacDonald (1975), among others. No consistent effects have been demonstrated for midwater fishes (see also Whitt and Prosser, 1971; Prosser *et al.*, 1975).

Previous studies on stress in marine teleosts have shown marked increases in osmolarity and chloride, but have failed to show significant changes in protein levels (Umminger, 1970a; Fletcher, 1974; Forster and Berglund, 1956). Stress increases renal urea excretion in elasmobranchs (Forster *et al.*, 1972), but no studies have demonstrated that stress changes blood urea levels in elasmobranchs or teleosts (review by Love, 1970). Among the constituents studied in only a few species, total carbohydrates are almost certainly elevated by stress (Chavin, 1964; Wedemeyer, 1972); phosphate may be affected by stress but the direction of the change is equivocal (Forster and Berglund, 1956; Hammond and Hickman, 1966); and little is known of how amino acids respond to acute stress in fishes.

Variation within groups

Shallow water teleosts. Blood sera of shallow water teleosts (Table I) are hyposmotic, with osmolarities (mean = 444; range = 333–587 mOsm/l) roughly 40% of sea water. All except the high values in *Makaira* and *Cheilopogon* and the low ones of *Opsanus* were close to the range (386–476 mOsm/l) compiled by Holmes and Donaldson (1969) for marine teleosts. Fletcher (1974), among others, has shown that post-mortem changes and stress can raise osmolarity in teleosts, which could account for the high values above. Serum chloride values (mean = 176; range = 139–234 m*M*/l) varied only slightly and were within the range of values compiled for marine teleosts by Holmes and Donaldson (1969) and Prosser (1973). The blood protein levels (mean = 5.0; range = 2.4–8.3 g/100 ml) were mostly in the range given by Holmes and Donaldson (1969) for comparable shallow water teleosts (2.8–8.0 g/100 ml). Serum urea levels (mean = 4.0; range = 1.3–10.4 m*M*/l) are low, as previously reported (Holmes and Donaldson, 1969).

Although TMAO in teleost serum has generally been regarded as negligible or undetectable (Norris and Benoit, 1945; Lange and Fugelli, 1965; Nicol, 1967), the present findings suggest it is a significant solute in the blood sera of several shallow water teleosts, including *Pomatomus, Stenotomus,* and *Cynoscion* (mean = 14; range = 1–37 m*M*/l). Diet, stress, season, or other factors may have contributed to these high values (Love, 1970).

The osmotic deficit was quite high for some species (*e.g.* over 100 m*M*/l in *Cheilopogon, Morone, Scomber,* and *Makaira*), suggesting they may contain substantial quantities of unidentified solutes. Teleost fishes, like tetrapods, often have large cation excesses (Pickford *et al.*, 1969) in which chloride levels are much lower than the levels of cations such as sodium and potassium because of large amounts of bicarbonate, phosphate, amino acids, lactate, and protein. The low osmotic deficit of *Stenotomus* (excess of 23 m*M*/l) may be an artifact of the very high TMAO concentration in the one specimen measured. In at least some shallow water teleosts, amino acids (mean = 12.4; range = 5–25 m*M*/l) may contribute substantially to osmolarity (Table V). Total carbohydrates (mean = 175; range = 54–385 mg/100 ml) probably are minor osmotic constituents in unstressed fish, and phosphate levels (mean = 5.6; range = 4.6–6.5 m*M*/l) also are relatively minor osmotically. The levels of amino acids, carbohydrates, and phosphate, except the very high carbohydrate concentration in *Pomatomus*, agree with published values in teleosts (summarized by Pickford *et al.*, 1969, and Griffith *et al.*, 1974).

Marine elasmobranchs. All of the elasmobranchs studied except *Centroscymnus* (Table II) had consistent osmolarities (991–1092 mOsm/l; mean = 1035) similar to that of sea water, chloride levels of 256–278 m*M*/l, high urea (368–416 m*M*/l), and protein levels of 1.7–3.9 g/100 ml. These data agree with most published reports on the blood chemistry of marine elasmobranchs (reviews by Smith, 1936; Holmes and Donaldson, 1969; Pang *et al.*, 1977). The high osmolarity and chloride and low protein and urea in *Centroscymnus* (Table II) may be an artifact of post-

TABLE V

Other blood serum constituents in some marine fishes. (Values are mean ± SE when N ≥ 3 and mean alone when N < 3. Sample size is in parentheses.)

Group/species	Amino acids (m*M*/l)	Carbohydrates (mg/100 ml)	Phosphate (m*M*/l)
Shallow Water Teleosts			
Brevoortia tyrannus	8.9 (2)	121 (2)	4.6 (1)
Gadus morhua	5.0 (1)	54 (1)	6.4 (1)
Pomatomus saltatrix	11.1 ± 1 (3)	385 ± 81 (5)	6.5 (1)
Sphyraena borealis	24.5 (2)	138 (2)	5.0 (1)
Elasmobranchs			
Raja erinacea	3.5 (1)	129 (1)	—
Squalus acanthias	4.2 (1)	—	—
Prionace glauca	—	133 (2)	—
Centroscymnus coelolepis	18.9 (1)	42 (1)	8.0 (1)
Benthic Teleosts			
Abyssal rattail	34.2 (1)	121 (1)	6.2 (1)
Nematonurus armatus	12.1 (2)	44 (2)	6.2 (2)
Urophycis tenuis	20.6 (2)	44 (2)	3.3 (2)
Midwater Teleosts			
Malocosteus niger	—	62 (1)	—
Chauliodus sloani	6.7 (1)	47 (1)	5.8 (1)
Anoplogaster cornuta	13.2 (1)	124 (1)	8.2 (1)
Scopelogadus beani	4.5 (2)	9 (2)	1.8 (1)

mortem changes or it may be due to a deep-sea habitat, since the deep water holocephalans have similar blood serum chemistry (Fänge and Fugelli, 1963; Robertson, 1976; Read, 1971a).

Reported serum TMAO levels of elasmobranchs are high (reviews by Pang *et al.*, 1977; Holmes and Donaldson, 1969; Love, 1970; Nicol, 1967; Forster and Goldstein, 1969). The levels in this study were substantial but varied (range = 22–99; mean = 66 m*M*/l). The elasmobranch osmotic deficits generally were low, from an excess of 41 m*M*/l in *Centroscymnus* to a deficit of 65 m*M*/l in *Squalus*, suggesting most elasmobranchs do not contain unidentified solutes in large amounts. Serum amino acids (mean = 8.9; range = 3.5–18.9 m*M*/l) and carbohydrates (mean = 101; range = 42–183 mg/100 ml) were relatively insignificant osmotically and agreed with published data (Keirmeir, 1939; Denis, 1922; Brull, 1956; Smith, 1929). *Centroscymnus* phosphate (8 m*M*/l; Table V) was higher than previously reported for other elasmobranchs (Holmes and Donaldson, 1969).

Deep benthic teleosts. Serum osmolarities in deep benthic teleosts studied here (Table III) range from unexceptional levels of around 486 in *Nezumia* and *Antimora* to very high (albeit hyposmotic to sea water) levels of 775 mOsm/l in *Bathypterois*. Similarly, chloride ranged from moderate values of *ca.* 200 m*M*/l in some species to 319 m*M*/l in *Bathypterois*. Urea levels (0.9–4.0; mean = 1.5 m*M*/l), and protein levels (2.0–2.9; mean = 2.4 g/100 ml) varied little. TMAO concentrations ranged from a low of 6.3 m*M*/l in the abyssal rattail to a high in *Antimora* of 97 m*M*/l. Published data on electrolytes in rattails (Fänge *et al.*, 1972; Whitt and Prosser, 1971; Prosser *et al.*, 1975) show values generally similar to those found here. Many benthic species examined here had large osmotic deficits (4 species over 100 m*M*/l), suggesting that their blood contained considerable unidentified solutes. The high amino acids in some species (mean = 22; range = 12–34 m*M*/l) may have contributed to the unexplained osmolarity. Carbohydrates (mean = 70; range = 44–101 mg/100 ml) and phosphate (mean = 5.2; range = 3.3–6.2 mM/l) were probably of less consequence (Table V).

Midwater teleosts. Osmolarity and chloride of midwater teleost fishes (Table IV) ranged widely (363–753; mean = 561 mOsm/l osmolarity), (184–352; mean = 267 mM/l chloride), although all species were hyposmotic and hypoionic to sea water. Blood urea, ranging from 0.1–1.9 mM/l (mean = 1.0 mM/l) was a somewhat variable but osmotically insignificant solute in all species tested, and blood proteins (0.3–1.9 g/100 ml; mean = 0.8 g/100 ml) tended to be low, though variable. Serum TMAO concentrations (0.0–35 mM/l; mean = 12 mM/l) were variable and were a consequential osmotic solute in some species such as *Echiostoma, Chauliodus, Gempylus,* and *Argyropelycus.* The osmotic deficit (−32 to 136 mM/l) was low in most species, indicating that the measured solutes gave a good picture of overall serum osmolarity. Serum amino acids (mean = 8.1; range = 6.7–13.2 mM/l), carbohydrates (mean = 61; range = 9–124 mg/100 ml), and phosphate (mean = 5.3; range = 1.8–8.2 mM/l) although measured in a few species (Table V), did not appear to be important osmotic solutes. Blaxter *et al.* (1971) indicate that midwater teleosts have a wide range of osmolarities (*ca.* 365–1000 mOsm/l, estimated from freezing point depression), with the highest value isosmotic with sea water. Blaxter *et al.* (1971) also point out that many midwater fishes, especially those lacking swim bladders, had high water content. This is consistent with the low serum protein levels reported here.

Comparison of serum solutes in the various groups of marine fishes

Osmolarity. The osmolarity of the blood serum of elasmobranch fishes (1035 mOsm/l, Table VI) was much higher than that found for the teleosts. It was consistently isosmotic or somewhat hyperosmotic to the sea water environment (generally *ca.* 1000 mOsm/l; Prosser, 1973). Within the teleosts, the osmolarities of both the midwater and deep benthic fishes were similar (561 and 576 mOsm/l, respectively), and higher, though not significantly so at the 5% confidence level, than those of the shallow water species (444 mOsm/l). The differences between the hyposmotic teleosts and the isosmotic elasmobranchs have been reviewed many times in the literature (Pang *et al.*, 1977; Smith, 1936; Smith, 1953; Holmes and Donaldson, 1969; Conte, 1969; etc.). It is questionable whether the trends found here, for the midwater and deep benthic fishes to have higher osmolarities than the shallow water species, are meaningful. The deep-sea fishes were mostly sampled while dead, whereas the shallow water fishes were mostly sampled alive, and post-mortem changes and stress probably lead to elevated blood osmolarities in teleosts. However, some midwater, deep benthic, and shallow water teleosts may possess fairly high osmolarities in the natural state, though none approach being isosmotic with the environment.

Chloride. Blood chloride levels were highest in elasmobranchs (295 mM/l). These elasmobranch levels were significantly higher than those of shallow water teleosts (176 mM/l), but did not differ significantly from those of the deep benthic (243 mM/l) or midwater (267 mM/l) teleosts (Table VI). None of the teleost groups differed significantly from one another, although the deep-sea groups tended to have higher chloride levels than the shallow water group. Elasmobranchs generally have higher serum ion levels than (shallow water) teleosts (reviews by Holmes and Donaldson, 1969; Pang *et al.*, 1977). The generally higher chloride levels in

TABLE VI

*A comparison of the blood serum composition of different groups of marine fishes. (Values are the means of the means for species from Tables I–V ± SE, with the ranges of species' means in parentheses. * = Significantly different from shallow water teleosts,* $P < .05$*, using Student's* t *test.)*

	Osmolarity (mOsm/l)	Chloride (m*M*/l)	Urea (m*M*/l)	Protein (g/100 ml)	TMAO (m*M*/l)	Amino Acids (m*M*/l)	Carbohydrates (mg/100 ml)	PO_4 (m*M*/l)
Shallow water teleosts	444 ± 19	176 ± 8	4.0 ± 0.7	5.0 ± 0.4	14.3 ± 3.0	12.4 ± 4.2	175 ± 73	5.6 ± 0.5
	(333–587)	(139–241)	(1.3–10.4)	(2.4–8.3)	(1.3–37.4)	(5.0–24.5)	(54–385)	(4.6–6.5)
Marine elasmobranchs	1035 ± 17*	295 ± 26*	363 ± 32*	2.5 ± 0.5	65.7 ± 12.0	8.9 ± 5.0	101 ± 30	8.0
	(991–1092)	(256–423)	(208–416)	(0.5–3.9)	(22–99)	(4.2–18.9)	(42–133)	
Deep benthic teleosts	576 ± 30	243 ± 16	1.5 ± 0.4	2.4 ± 0.2	50.7 ± 26.2	22.3 ± 6.4	70 ± 26	5.2 ± 1.0
	(487–775)	(171–319)	(0.7–4.0)	(2.0–2.9)	(6.3–97)	(12.1–34.2)	(44–121)	(3.3–6.2)
Midwater teleosts	561 ± 22	267 ± 12	1.0 ± 0.1	0.8 ± 0.1*	12.1 ± 2.5	8.1 ± 2.6	61 ± 24	5.3 ± 1.9
	(363–753)	(184–352)	(0.1–2.3)	(0.3–1.9)	(0.0–35.0)	(6.7–13.2)	(9–124)	(1.8–8.2)

deep-sea fishes may indicate that higher blood ions conserve energy, or may be an artifact of trawling stress and post-mortem changes.

Urea. Only elasmobranchs, among the fishes studied, had high urea levels (363 m*M*/l; Table VI). All marine elasmobranchs retain high levels of urea (Smith, 1936; Pang *et al.*, 1977; Holmes and Donaldson, 1969). This is also characteristic of holocephalans (Read, 1971a; Fänge and Fugelli, 1963; Robertson, 1976) and the coelacanth (Pickford and Grant, 1967; Lutz and Robertson, 1971; Griffith, 1980). In these groups urea brings osmolarity close to that of sea water while maintaining blood electrolytes at levels much lower than sea water. No teleost species studied in the present report had urea levels high enough to act as a substantial osmotic solute. Deep water teleosts may have lower urea levels than shallow water species, although the differences were not statistically significant.

Protein. Serum protein levels tended to be highest in shallow water teleosts, intermediate in elasmobranchs and deep benthic teleosts, and lowest in midwater teleosts (Table VI). Protein in the midwater teleosts was much lower than in inshore teleosts, but no other difference between groups in serum protein levels was significant. The low protein levels in midwater teleosts may be due to their need to maintain neutral buoyancy with minimal energy, resulting in reduction of hard skeletal material and heavy proteins. Such reduction in midwater fishes, especially those lacking a gas bladder, has been documented by Denton and Marshall (1958) and Blaxter *et al.* (1971). The lower serum protein levels in elasmobranchs and deep benthic teleosts than in shallow water teleosts are probably not related to buoyancy. These levels may relate to activity, since within the shallow water teleosts, active fish tend to have higher serum protein than inactive fish like *Opsanus*.

Trimethylamine oxide. Serum TMAO levels varied within all four groups of fishes studied, and comparisons among the groups showed no significant differences (Table VI). Most elasmobranchs, however, had high TMAO levels, consistent with the osmoregulatory role usually ascribed to TMAO in these fishes (reviews by Smith, 1936; Groninger, 1959; Pang *et al.*, 1977). A number of teleosts also had high TMAO levels that presumably contribute substantially to their total serum osmolarity. No clear relationships between habitat and TMAO concentrations were evident. Differences in diet might account for some of the variability, since diet is teleosts' principal source of TMAO (Norris and Benoit, 1945).

Amino acids, carbohydrates, and phosphate. Differences in serum amino acids, carbohydrates, and inorganic phosphate levels were neither consistent nor significant in the four groups studied (Tables V and VI), although too few species for generalization may have been tested. In certain species, amino acids contributed substantially to osmolarity (*e.g.*, levels over 15 mM/l in *Sphyraena, Centroscymnus, Urophycis*, and the abyssal rattail), but the contributions of carbohydrates (a maximum of *ca.* 21 mM/l in the hyperglycemic *Pomatomus*, and probably less in unstressed animals) and inorganic phosphate (the highest was 8.2 mM/l) were probably not important to total serum osmolarity.

Hematocrits. Hematocrits in the few inshore teleosts we measured, mostly active species such as *Scomber, Coryphaena,* and *Carynx*, were around 50%. Hematocrits in elasmobranchs were lower (5–29%), in agreement with Wintrobe (1934). The deep benthic teleosts had values of 15–40% (average = 25%), and the lowest hematocrits were in the midwater teleosts (2–30%, but none over 10% except *Argyropelycus* and the myctophid species studied). Although stress and morbidity affect hematocrit, the change usually is an increase (Fletcher, 1974), suggesting that our low values are realistic. Blaxter *et al.* (1971) observed similar low hematocrits (5–9%) in midwater fishes lacking swim bladders, and suggested that this was due to low activity.

Discussion

Griffith *et al.* (1973) suggested that deep-sea teleosts evolved urea retention to conserve energy in osmoregulation. The present data do not support this hypothesis. No midwater or deep benthic teleost investigated had levels of urea above 4 mM/l; most had less than shallow water teleosts. Although Griffith *et al.* (1973) further suggested that urea retention would be even more advantageous to viviparous or ovoviviparous deep-sea fishes such as brotulids or zoarcids, the abyssal brotulid tested had low urea (0.7 mM/l) and the serum of shallow water *Macrozoarces americanus*, an oviparous relative of the viviparous zoarcids, also had negligible urea concentrations (Griffith, unpublished). In light of the large number of species from a variety of habitats found to lack the mechanism, it seems likely that no teleost possesses ureosmotic regulation.

One alternative to the urea retention hypothesis is that some deep-sea teleosts could build up ion (NaCl) concentrations to sea water levels and conserve energy in both osmotic and ionic regulation. Although high osmolarity, mostly accounted for by chloride (plus sodium), exists in a variety of deep-sea fishes, no teleost studied reached the isosmotic, isoionic condition found in hagfishes. The highest teleostean osmolarities and chloride levels found here were 775 mOsm/l and 319 mM/l in the benthic *Bathypterois*, and 753 mOsm/l and 331 mM/l in the midwater eel, *Derichthys*. These contrast with the hagfish, *Myxine*, with an osmolarity of 1034 mOsm/l and chloride levels of 508 mM/l (Robertson, 1966; Munz and McFarland, 1964). Some deep-sea teleosts may be evolving the hagfish mechanism, but the generally poor physiological condition of all deep-sea fishes studied and the fact that trawling stress and morbidity elevate chloride and osmolarity in marine teleosts (Fletcher, 1974; Umminger, 1970a; Forster and Berglund, 1956) make the results of the present study unclear. The lowest osmolarities and chloride levels in midwater fish specimens were from those in the best physiological condition, a finding also evident from Blaxter *et al.* (1971).

Deep-sea fishes could elevate blood osmolarity without raising blood electrolytes to toxic levels nor developing special mechanisms to retain urea, by building up organic solutes such as amino acids, carbohydrates, or other metabolic products. The best osmotic solutes (1) would be non-toxic, readily supplied through common metabolic pathways or the diet, (2) should not be crucial to energy or synthetic metabolism, and (3) should not pass easily through membranes between the fish and its environment. TMAO, retained in addition to urea in marine elasmobranchs, is one example of this sort. Although vertebrates' use of amino acids or carbohydrates as extracellular solutes in osmoregulation has not been documented, many invertebrates and some vertebrates do build up these compounds in response to low temperatures (Umminger, 1970b; Prosser, 1973).

The present data suggest some deep-sea teleosts might use TMAO and amino acids to elevate osmolarity. Among benthic deep-sea teleosts high TMAO (over 40 m*M*/l) is found in *Antimora* and *Nematonurus*, and high amino acids (over 20 m*M*/l) in *Urophycis* and an abyssal rattail. Four species (*Bathypterois, Lepidophidium, Macrozoarces*, and an abyssal brotulid) have osmotic deficits of more than 100 m*M*/l, suggesting abundant non-measured solutes that may include TMAO and amino acids. Among midwater fishes these compounds seem to be less important, most species having low osmotic deficits and only a few having osmotically significant levels of TMAO or amino acids. A variety of shallow water teleosts have substantial osmotic deficits, TMAO levels, or amino acids. No species studied, however, had levels of extra solutes high enough to bring the total osmolarity close to that of sea water. Stress and morbidity might break down the cellular barrier, releasing TMAO, amino acids, lactate, etc., and thus raising concentrations of these substances in the blood. Hence, this hypothesis is dubious and of limited importance.

Blaxter *et al.* (1971) suggested that midwater fishes, particularly those lacking swim bladders, might reduce total solute concentrations in their body fluids to help achieve neutral buoyancy. The present data confirm the results of Blaxter *et al.* (1971), but fail to substantiate their hypothesis. The midwater fishes studied here generally had higher total solutes, as reflected in total osmolarity, than did shallow water teleosts. The buoyancy hypothesis, however, may partially explain why midwater fishes have few red blood cells and low concentrations of particularly heavy solutes, such as proteins. The need for neutral buoyancy might have inhibited midwater fishes from developing osmoregulatory specializations that involve accumulating extra solutes.

Thus, deep-sea teleosts appear to osmoregulate by the same mechanisms used by their shallow water relatives. Although adaptation to the deep-sea environment resulted in the evolution of extraordinary physiological and/or morphological specializations in nearly every functional system in some fishes (general reviews by Gordon, 1970; Brauer, 1972; MacDonald, 1975; and Locket, 1977), no such interesting specializations have developed in teleostean osmoregulation. The reasons that novel mechanisms did not develop are probably complex and may involve such factors as buoyancy. However, once vertebrates acquired low blood electrolytes and once teleosts had adapted to marine environments by hyposmotic rather than ureosmotic regulation, changing to another osmoregulatory strategy may have become impossible.

ACKNOWLEDGMENTS

This study would not have been possible without the aid and encouragement of many individuals associated with Woods Hole Oceanographic Institution. I am especially indebted to the officers, crew, chief scientists, and scientific personnel who served on cruises AII-85, Knorr-47, Chain 123, and Chain 125 for enabling me to collect blood samples from deep-sea fishes. Blood sera from a few additional specimens was provided by Drs. Richard Haedrich and Tracy McClellan. Laboratory facilities at W.H.O.I. were provided by Drs. Frank Carey, John Stegeman, and Dennis Sabo. Financial support was provided by the W.H.O.I. education department through a postdoctoral scholar award. I am grateful to Drs. Frank Carey, John Stegeman, and Sanford Moss for reading the manuscript and giving helpful comments.

LITERATURE CITED

Blaxter, J. H. S., C. S. Wardle, and B. L. Roberts. 1971. Aspects of the circulatory physiology and muscle systems of deep-sea fish. *J. Mar. Biol. Assoc. U. K.,* **51:** 991–1006.

Brauer, R. W. (Ed.), 1972. *Barobiology and the Experimental Biology of the Deep Sea.* North Carolina Sea Grant Program, Univ. of North Carolina, Chapel Hill. 428 pp.

Brown, G. W., and P. P. Cohen. 1960. Comparative biochemistry of urea synthesis 3. Activities of urea-cycle enzymes in various higher and lower vertebrates. *Biochem. J.* **75:** 82–91.

Brull, L. 1956. L'azote non-proteique du plasma de *Scylliorhinus canicula. Vie et Millieu,* **7:** 521–522.

Chavin, W. 1964. Sensitivity of fish to environmental alterations. *Proc. 7th Conf. Great Lakes Res., Univ. Mich.* Great Lakes Res. Div. Publ. **11:** 54–67.

Conte, F. P. 1969. Salt secretion. Pages 241–292 in W. S. Hoar and D. J. Randall, Eds., *Fish Physiology.* Volume I. Academic Press, New York.

Denis, W. 1922. The non-protein organic constituents in the blood of marine fish. *J. Biol. Chem.* **54:** 693–700.

Denton, E. J., and N. B. Marshall. 1958. The buoyancy of bathypelagic fishes without a gas-filled swim bladder. *J. Mar. Biol. Assoc. U. K.* **37:** 753–767.

Fänge, R., and K. Fugelli. 1963. The rectal salt gland of elasmobranchs and osmoregulation in chimaeroid fishes. *Sarsia* **10:** 27–34.

Fänge, R., A. Larrson, and U. Lidman. 1972. Fluids and jellies of the acustico-lateralis system in relation to body fluids in *Coryphaenoides rupestris* and other fishes. *Mar. Biol.* **17:** 180–185.

Fletcher, G. 1974. The effects of capture, "stress," and storage of whole blood on the red blood cells, plasma proteins, glucose, and electrolytes of the winter flounder (*Pseudopleuronectes americanus*). *Can. J. Zool.* **53:** 197–206.

Forster, R. P., and F. Berglund. 1956. Osmotic diuresis and its effect on total electrolyte distribution in plasma and urine of the aglomerular teleost, *Lophius americanus. J. Gen. Physiol.* **39:** 349–359.

Forster, R. P., F. Berglund, and B. R. Rennick. 1958. Tubular secretion of creatine, trimethylamine oxide and other organic bases by the aglomerular teleost, *Lophius americanus. J. Gen. Physiol.* **42:** 319–327.

Forster, R. P., and L. Goldstein. 1969. Formation of excretory products. Pp. 313–350 in W. S. Hoar and D. J. Randall, Eds., *Fish Physiology,* Volume I. Academic Press, New York.

Forster, R. P., L. Goldstein, and J. K. Rosen. 1972. Intrarenal control of urea reabsorption by renal tubules of the marine elasmobranch, *Squalus acanthias. Comp. Biochem. Physiol.* **42A:** 3–12.

Gordon, M. S. 1970. Hydrostatic pressure. Pp. 445–464 in W. S. Hoar and D. J. Randall, Eds., *Fish Physiology,* Volume IV. Academic Press, New York.

Griffith, R. W. 1980. Chemistry of the body fluids of the coelacanth, *Latimeria chalumnae. Proc. R. Soc. Lond. B. Biol. Sci.* **208:** 329–347.

Griffith, R. W., and P. K. T. Pang. 1979. Mechanisms of osmoregulation in the coelacanth: Evolutionary implications. *Occ. Pap. Calif. Acad. Sci.* **134:** 79–93.

Griffith, R. W., P. K. T. Pang, and L. A. Benedetto. 1979. Urea tolerance in the killifish, *Fundulus heteroclitus. Comp. Biochem. Physiol.,* **62A:** 327–330.

Griffith, R. W., P. K. T. Pang, A. K. Srivastava, and G. E. Pickford. 1973. Serum composition of freshwater stingrays (Potamotrygonidae) adapted to fresh and dilute sea water. *Biol. Bull.* **144:** 304–320.

Griffith, R. W., B. L. Umminger, B. F. Grant, P. K. T. Pang, and G. E. Pickford. 1974. Serum composition of the coelacanth, *Latimeria chalumnae* Smith. *J. Exp. Zool.* **187:** 87–102.

GRONINGER, H. S. 1959. Occurrence and significance of trimethylamine oxide in marine animals. *U. S. Fish Wildlife Serv. Spec. Sci. Rep. Fish.* **333:** 1–22.

HAMMOND, B. R., AND C. P. HICKMAN. 1966. The effect of physical conditioning on the metabolism of lactate, phosphate, and glucose in the rainbow trout, *Salmo gairdneri. J. Fish. Res. Bd. Can.* **23:** 65–83.

HARVEY, G. R., AND W. STEINHAUER. 1976. Biogeochemistry of PCB and DDT in the North Atlantic. Pp. 203–221 in J. O. Nriagu, Ed., *Environmental Biochemistry. Vol. I. Carbon, Nitrogen, Phosphorus, Sulfur and Selenium Cycles.* Ann Arbor Science Publ., Ann Arbor, Michigan.

HOLMES, W. N., AND E. M. DONALDSON. 1969. The body compartments and the distribution of electrolytes. Pp. 1–89 in W. S. Hoar and D. J. Randall, Eds., *Fish Physiology*, Volume I. Academic Press, New York.

HUGGINS, A. K., G. SKUTCH, AND E. BALDWIN. 1969. Ornithine-urea cycle enzymes in teleost fishes. *Comp. Biochem. Physiol.* **28:** 587–602.

JAHN, A. E. 1976. *On the midwater fish faunas of Gulf Stream rings with respect to habitat differences between Slope water and northern Sargasso Sea.* Ph.D. Dissertation, Woods Hole Oceanographic Institution. Woods Hole, Massachusetts, 173 pp.

KIERMEIR, A., 1939. Über den blutzucher der süsswasser fische. *Z. Vergl. Physiol.* **27:** 460–491.

LANGE, R., AND K. FUGELLI. 1965. The osmotic adjustment in the euryhaline teleosts, the flounder, *Pleuronectes flesus* L. and the three-spined stickleback, *Gasterosteus aculeatus* L. *Comp. Biochem. Physiol.* **15:** 283–292.

LOCKET, N. A. 1977. Adaptations to the deep-sea environment. Pp. 67–192 in F. Crescitelli, Ed., *Handbook of Sensory Physiology* Volume VII/5, (The visual system in vertebrates). Springer-Verlag, Berlin.

LOVE, R. M. 1970. *The Chemical Biology of Fishes.* Academic Press, New York. 547 pp.

LOWRY, O. H., N. R. ROBERTS, K. Y. LEINER, M. L. WU, AND A. L. FARR. 1953. The quantitative histochemistry of brain I. Chemical methods. *J. Biol. Chem.* **208:** 1–17.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–276.

LUTZ, P. L., AND J. D. ROBERTSON. 1971. Osmotic constituents of the coelacanth, *Latimeria chalumnae* Smith. *Biol. Bull.* **141:** 553–560.

MACDONALD, A. G. 1975. *Physiological Aspects of Deep-Sea Biology.* Monographs of the Physiological Society No. 31. Cambridge Univ. Press, Cambridge. 450 pp.

MUNZ, F. W., AND W. N. MCFARLAND. 1964. Regulatory function of a primitive vertebrate kidney. *Comp. Biochem. Physiol.* **13:** 381–400.

NICOL, J. A. C. 1967. *The Biology of Marine Animals.* Wiley-Interscience, New York. 707 pp.

NORRIS, E. R., AND G. J. BENOIT. 1945. Studies on trimethylamine oxide. I. Occurrence of trimethylamine oxide in marine organisms. *J. Biol. Chem.* **158:** 433–438.

PANG, P. K. T., R. W. GRIFFITH, AND J. W. ATZ. 1977. Osmoregulation in elasmobranchs. *Amer. Zool.* **17:** 365–377.

PICKFORD, G. E., AND F. B. GRANT. 1967. Serum osmolality in the coelacanth, *Latimeria chalumnae*: Urea retention and ion regulation. *Science* **155:** 568–570.

PICKFORD, G. E., F. B. GRANT, AND B. L. UMMINGER. 1969. Studies on the blood serum of the euryhaline cyprinodont fish, *Fundulus heteroclitus*, adapted to fresh or to salt water. *Trans. Conn. Acad. Arts Sci.* **43:** 25–70.

PROSSER, C. L. (Ed.), 1973. *Comparative Animal Physiology.* Third Edition. W. B. Saunders Co., Philadelphia. 966 pp.

PROSSER, C. L., W. WEEMS, AND R. MEISS. 1975. Physiological state, contractile properties of heart and lateral muscles of fishes from different depths. *Comp. Biochem. Physiol.* **52B:** 127–131.

RASMUSSEN, L., AND R. A. RASMUSSEN. 1977. Exogenous ^{14}C-urea distribution in selected marine fishes—especially two species of salmon and resultant alteration in sera and gill enzyme levels. *Comp. Biochem. Physiol.* **56A:** 403–409.

READ, L. J. 1967. Enzymes of the ornithine-urea cycle in the chimaera *Hydrolagus colliei. Nature* **215:** 1412–1413.

READ, L. J. 1971a. Chemical constituents of the body fluids and urine of the holocephalian *Hydrolagus colliei. Comp. Biochem. Physiol.,* **39A:** 185–192.

READ, L. J. 1971b. The presence of high ornithine-urea cycle enzyme activity in the teleost, *Opsanus tau. Comp. Biochem. Physiol.* **39A:** 409–413.

ROBERTSON, J. D. 1966. Osmotic constituents of the blood plasma and parietal muscle of *Myxine glutinosa.* Pp. 631–644 in H. Barnes, Ed., *Some Contemporary Studies in Marine Science.* Allen and Unwin, London.

ROBERTSON, J. D. 1976. Chemical composition of the body fluids and muscle of the hagfish *Myxine glutinosa* and the rabbit-fish *Chimaera monstrosa. J. Zool. Lond.* **178:** 261–277.

Smith, H. W. 1929. The composition of the body fluids of elasmobranchs. *J. Biol. Chem.* **81:** 407–419.

Smith, H. W. 1936. The retention and physiological role of urea in the Elasmobranchii. *Biol. Rev.* **11:** 49–92.

Smith, H. W. 1953. *From Fish to Philosopher.* Little Brown and Co., Boston. 264 pp.

Troll, W., and R. K. Cannon. 1953. A modified photometric ninhydrin method for the analysis of amino and imino acids. *J. Biol. Chem.* **200:** 803–811.

Umminger, B. L. 1970a. Effects of subzero temperatures and trawling stress on serum osmolality in the winter flounder, *Pseudopleuronectes americanus. Biol. Bull.* **139:** 574–579.

Umminger, B. L. 1970b. Physiological studies on supercooled killifish (*Fundulus heteroclitus*) III. Carbohydrate metabolism and survival at subzero temperatures. *J. Exp. Zool.* **173:** 159–174.

Wedemeyer, G. 1972. Some physiological consequences of handling stress in the juvenile coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Salmo gairdneri*). *J. Fish Res. Bd. Can.* **29:** 1780–1783.

Whitt, G. S., and C. L. Prosser. 1971. Lactate dehydrogenase isozymes, cytochrome oxidase activity and muscle ions of the rattail (*Coryphaenoides* sp.), *Amer. Zool.* **11:** 503–511.

Wintrobe, M. M. 1934. Variations in the size and hemoglobin content in the blood of various vertebrates. *Folia Haem.* **51:** 32–49.

Reference: *Biol. Bull.*, **160:** 265–271. (April, 1981)

METABOLIC SIGNIFICANCE OF THE DIETARY CMP REQUIREMENT IN RELATION TO *ARTEMIA* MORPHOGENESIS

A. HERNANDORENA

Laboratoire du Muséum National d'Histoire Naturelle, C.E.R.S. 64200 Biarritz (France)

Abstract

In *Artemia*, the effect of actinomycin D, which inhibits RNA synthesis, and the effect of puromycin, which inhibits protein synthesis, depend on the AMP:CMP ratio of the diet. When RNA synthesis is limited by CMP concentrations deficient in relation to AMP, protein synthesis decreases and the abdominal length of *Artemia* increases. When RNA synthesis is limited by AMP concentrations deficient in relation to CMP, protein synthesis increases and *Artemia* abdominal segments develop supernumerary gonopodes. Induction of supernumerary gonopodes by a reduced AMP:CMP ratio is suppressed by an albumin deficiency and by puromycin. These results suggest that supernumerary gonopodes are induced by a reduced RNA:protein ratio.

Introduction

The artificial medium proposed by Provasoli and d'Agostino (1969) for the axenic culture of *Artemia* contains a mixture of adenylic acid, guanylic acid, cytidylic acid, uridylic acid, and thymidine. The purine and pyrimidine requirement of *Artemia* can be satisfied by adenylic acid and cytidylic acid; the quantitative adenylic acid requirement depends on the cytidylic acid concentration of the diet and vice-versa. Thymidine is not essential in a medium containing folic acid, provided the cytidylic acid is not limiting in relation to the adenylic acid concentration in the medium (Hernandorena, 1979). The ratio between dietary adenylic acid and cytidylic acid concentrations modifies not only growth and survival rates of larvae but also their morphogenesis. A dietary adenylic-acid deficiency induces supernumerary gonopodes on abdominal segments (Hernandorena, 1970), but not if the medium is lacking pyrimidines (Hernandorena, 1977). The same supernumerary gonopodes are induced by excess cytidylic acid in a medium containing the standard adenylic-acid concentration, but not if the medium contains excess adenylic acid (Hernandorena 1977, 1979). Thus, the morphogenetic action results from a reduced adenylic acid:cytidylic acid ratio.

Hernandorena (1975) examined the metabolic significance in nucleic acid metabolism and protein synthesis of the dietary adenylic acid requirement, using a medium containing a standard pyrimidines supply (cytidylic acid 10 mg %, uridylic acid 10 mg %, thymidine 5 mg %). In these conditions, effects of actinomycin D decrease with increasing adenylic acid concentration up to 140 mg %, and effects of puromycin decrease with increasing adenylic acid concentration up to 100 mg %. The present report is concerned with the metabolic significance of the dietary adenylic acid:cytidylic acid ratio.

Received 29 July 1980, accepted 13 January 1981.

MATERIALS AND METHODS

The method developed by Provasoli and d'Agostino (1969) for axenic culture of the *Artemia* Utah strain was used. Dietary purine requirement was met by adenylic acid (AMP) and pyrimidine requirement by cytidylic acid (CMP). Albumin concentration was 20 mg % unless otherwise stated. The antibiotics used were from Nutritional Biochemical Co. Rearing temperature was 25° ± 0.5°, salinity 24‰; 10 ml of medium was used to rear five animals, and 50–100 larvae were reared for each experimental conditions. Growth is estimated by a numerical index defined by Provasoli and d'Agostino (1969). This growth index approximates number of molts and size. For graphic comparisons of the experiments, the growth index for the 14th day of development was reported.

RESULTS

Results are summarized graphically. Figure 1 shows that the effect of actinomycin D, an inhibitor of RNA synthesis, decreased with increasing CMP concentration provided AMP was more than 20 mg %. RNA synthesis was not limited by CMP with a 20 mg % AMP concentration. RNA synthesis was limited by CMP concentrations of 0–40 mg % with a 40 mg % AMP concentration, of 0–60 mg % with a 60 mg % AMP concentration, of 0–100 mg % with a 100 mg % AMP concentration. The CMP concentrations inducing supernumerary gonopodes are exactly those found to exceed the minimum CMP requirement for maximum RNA

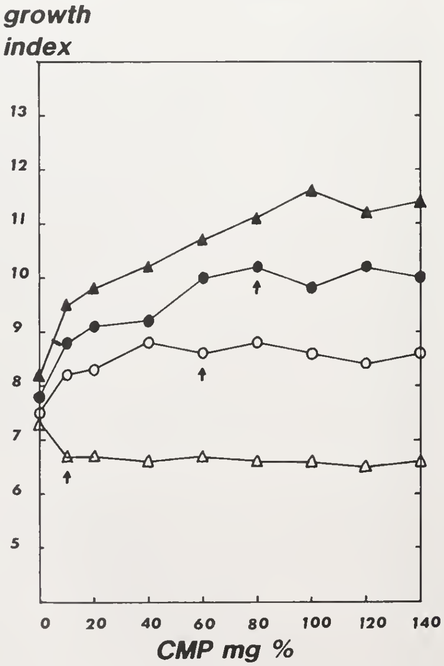
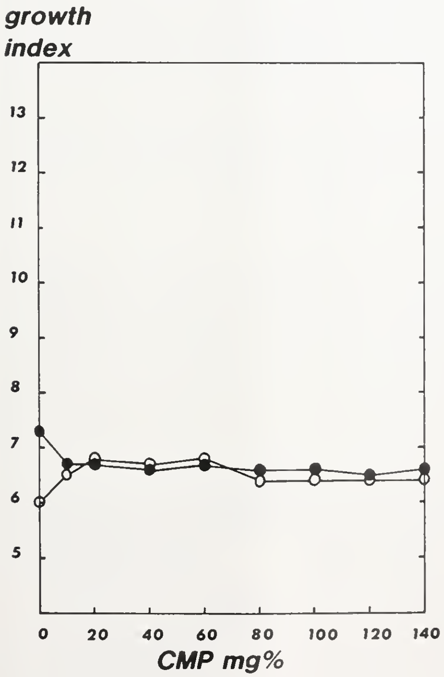


FIGURE 1. Effect on growth of actinomycin D (2 μg %) in relation to the AMP:CMP ratio. Open triangles = AMP: 20 mg %; Open circles = AMP: 40 mg %. Filled circles = AMP: 60 mg %; Filled triangles = AMP: 100 mg %. The arrows indicate minimum CMP concentrations inducing supernumerary gonopodes.

synthesis (arrows on Fig. 1). The threshold increased with increasing AMP concentrations.

The effect of puromycin, an inhibitor of protein synthesis, also depended on the AMP:CMP ratio of the diet (Fig. 2–5). With a 60 mg % AMP concentration, puromycin action decreased with increasing CMP concentration above the concentration found necessary for maximum RNA synthesis. Excess CMP did not induce supernumerary gonopodes in the presence of the antibiotic. With apparently the same RNA synthesis, more proteins were synthesized with a reduced AMP:CMP ratio than with a 60:60 ratio (Fig. 4). Similarly, more proteins were synthesized with a reduced ratio than with a 40:40 ratio (Fig. 3). At AMP concentrations of 100 mg %, excess CMP does not induce supernumerary gonopodes (Hernandorena, 1979). In this case, effects of both actinomycin D and puromycin decreased with increasing CMP concentrations (Fig. 5). With a 20 mg % AMP concentration, actinomycin D and puromycin were detrimental. Supernumerary gonopodes were induced provided the medium was not lacking pyrimidines (Hernandorena, 1977). Lack of pyrimidines, which did not decrease RNA synthesis, decreased protein synthesis (Fig. 2).

According to these results, the supernumerary gonopodes were induced by increased protein synthesis. If this is the case, an albumin deficiency or an albumin excess, which both increase the detrimental effect of puromycin (Hernandorena, 1975) should suppress the induction of supernumerary gonopodes in larvae reared in a medium containing excess cytidylic acid in relation to the adenylic acid con-



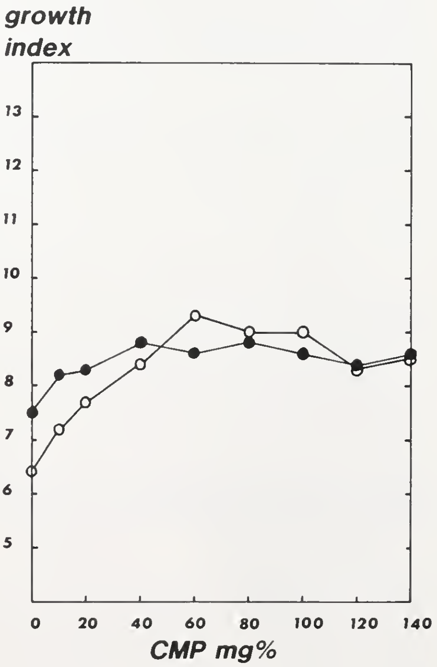
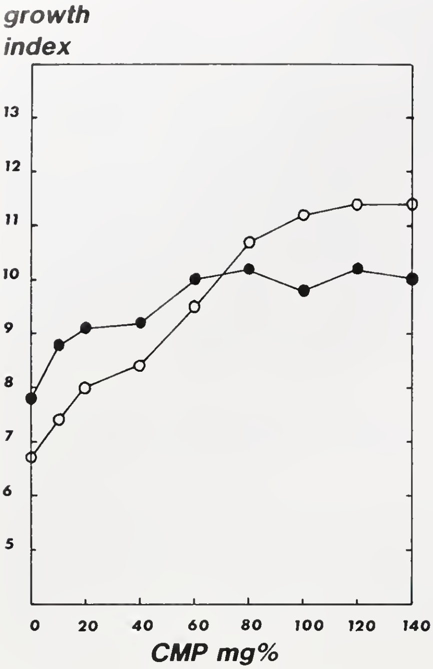


FIGURE 2. (Left.) Effect on growth of actinomycin D (2 μg %) (filled circles) and of puromycin (200 μg %) (open circles) in relation to the AMP:CMP ratio. AMP constant at 20 mg %.

FIGURE 3. (Right.) Effect on growth of actinomycin D (2 μg %) (filled circles) and of puromycin (200 μg %) (open circles) in relation to the AMP:CMP ratio. AMP constant at 40 mg %.



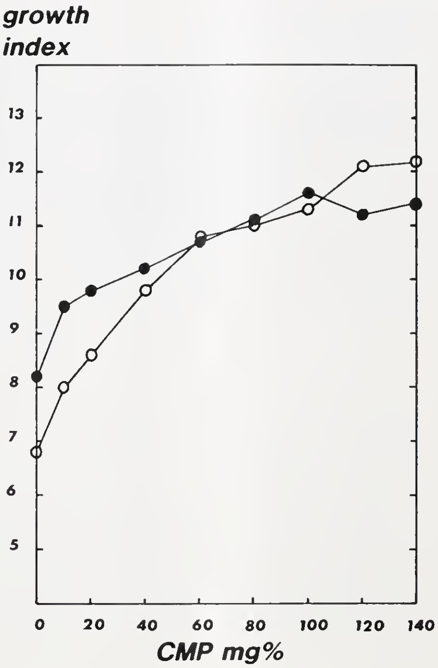


FIGURE 4. (Left.) Effect on growth of actinomycin D (2 μg %) (filled circles) and of puromycin (200 μg %) (open circles) in relation to the AMP:CMP ratio. AMP constant at 60 mg %.

FIGURE 5. (Right.) Effect on growth of actinomycin D (2 μg %) (filled circles) and of puromycin (200 μg %) (open circles) in relation to the AMP:CMP ratio. AMP constant at 100 mg %.

centration. As seen in Table I, an albumin deficiency but not an albumin excess suppressed the morphogenetic action of a reduced AMP:CMP ratio.

DISCUSSION

The results obtained by studying the effects of actinomycin D and of puromycin in relation to the AMP:CMP ratio in the diet suggest that the supernumerary gonopodes induced in larvae reared in a medium containing a reduced AMP:CMP ratio result from a reduced RNA:protein ratio.

Artemia, being incapable of *de novo* synthesis of the purine ring (Clegg *et al.*,

TABLE I

Effects of a reduced AMP:CMP ratio in relation to the albumin concentration of the diet.

AMP = 60 mg % CMP = 100 mg %	Growth index 14th day	Larval survival[1] (percentage)	Morphogenesis of appendages
Albumin mg %			
5	9.6	90	normal
20	11.0	80	supernumerary gonopodes
60	9.5	60	supernumerary gonopodes

[1] Percentage of larvae reaching growth index 10, independent of their growth rate.

1967; Warner and McClean, 1968) requires a dietary source of purine. *Artemia* can synthesize the pyrimidine ring from the usual precursors (Warner and McClean, 1968) but apparently at a limiting rate under our experimental conditions since growth rate and surviving numbers of larvae increase with increasing CMP dietary supply, provided adenylic acid is not limiting (Hernandorena, 1979). Our present results show that the CMP requirement for RNA synthesis depends on the AMP concentration. Consequently, the metabolic fate of CMP should depend on the AMP concentration. Protein synthesis is known to be an expensive chemical work in terms of ATP cost. With a given AMP concentration, excess CMP could contribute to increased energy production for "extra" protein synthesis.

An enzyme that converts CMP to CDP-choline is present in microsomal preparations of *Artemia* larvae (Ewing and Finamore, 1970a). This reaction could explain the increased energy production resulting from excess CMP. CDP-choline is used for phosphatidyl choline (lecithin) synthesis, and the rate of CDP-choline production from phosphatidyl choline is about twice the rate of synthesis of phosphatidyl choline from CDP-choline. The reversibility of the choline phosphotransferase reaction may be unique to *Artemia*. "This could be a means of energy conservation since the amount of energy invested in the activation of the choline moiety for synthesis of phosphatidyl choline could be salvaged as CDP-choline during degradation and reorganization of membrane structures" (Ewing and Finamore, 1970b).

Present and previous results strengthen this interpretation of the metabolic significance of a reduced AMP:CMP ratio. Induction of supernumerary gonopodes is associated with a reduced abdominal length (Hernandorena, 1972, 1975). Before my 1977 publication, the standard pyrimidine supply was used and the AMP:CMP ratio was increased by increasing AMP concentration. The abdominal length of *Artemia* increases with increasing AMP concentration in the diet. Increasing salinity has the same morphogenetic effect on larvae reared with a standard AMP concentration (Hernandorena, 1975). The effect of puromycin increases with increasing salinity both in nauplii (Ewing *et al.*, 1972) and during larval growth (Hernandorena, 1975). Conditions which limit the rate of protein synthesis, *i.e.*, albumin deficiency or increased salinity, suppress the induction of supernumerary gonopodes on larvae reared in an adenylic acid-deficient medium containing the standard pyrimidine supply (Hernandorena, 1974).

Present results showed that with a high AMP:CMP ratio, protein synthesis decreased, and that an albumin deficiency also suppressed the induction of supernumerary gonopodes on larvae reared in a cytidylic-acid-rich medium. Thus a reduced AMP:CMP ratio induces a morphogenetic action provided protein synthesis is not limited by an albumin deficiency. Albumin deficiency and excess both reduced growth rate; and, as already mentioned (Hernandorena 1975, 1977), the induction of supernumerary gonopodes is independent of larval growth rate but associated with a reduced survival. Albumin deficiency, which suppressed the morphological action, also increased the percentage of surviving larvae, while an albumin excess did not do so (Table I).

Increased protein synthesis resulting from excess CMP in relation to AMP concentration is further supported by the effect of dietary lecithin. Dietary lecithin raises the threshold of AMP concentrations inducing supernumerary gonopodes from 20 mg % to 40 mg % in larvae reared in a medium containing the standard pyrimidines supply (Hernandorena, 1976).

If the above interpretation is correct, a problem remains: How can quantitative differences in the rate of protein synthesis modify the competence of abdominal segments to develop supernumerary gonopodes and modify the abdominal length of *Artemia*? During *Acetabularia mediterranea* morphogensis, inhibition of protein synthesis by puromycin induces a loss of morphogenetic capacities (Brachet *et al.*, 1964). In *Artemia*, puromycin suppressed the induction of supernumerary gonopodes. Comparative nutritional data suggest that we are dealing with fundamental processes. In *Drosophila*, the same AMP:CMP ratio is involved in tumorigenesis (Sang and Burnet, 1963a) and in the expression of the eyeless phenotype (Sang and Burnet 1963b). Interestingly, the expression of these same mutant genes is altered by juvenile hormone mimics (Bryant and Sang, 1968). Dietary lecithin is also implicated in tumorigenesis (Sang and Burnet, 1967). Tumor formation in *Musca domestica* is related directly to the animal's ability to synthesize proteins (Bodnaryk, 1968). In *Drosophila* abdomens, the penetrance and expressivity of the abnormal mutation (A^{53g}) can be reduced by adding inhibitors of protein synthesis, RNA synthesis, and oxidative phosphorylation to the diet. The metabolic inhibitors that reduce the expressivity of the mutation also lower the protein concentration of the mutant flies. Adding adenosine to a cytidine-rich, adenosine-deficient diet increases both penetrance and expressivity to a normal level. The details on the effect of the abnormal abdomen genotype remain unknown (Hillman *et al.*, 1973).

In *Artemia*, the effect of RNA-synthesis limitation on protein synthesis and consequently on morphogenesis depends on the metabolic cause of the limitation: AMP deficiency and CMP deficiency have opposite effects. Further biochemical and nutritional investigations are needed.

LITERATURE CITED

Bodnaryk, R. P. 1968. Dietary amino acid and nucleic acid imbalance leading to altered tumor frequency in the adult housefly *Musca domestica. J. Insect. Physiol.* **14:** 223–242.

Brachet, J., H. Denis, and F. de Vitry. 1964. The effects of actinomycin D and puromycin on morphogenesis in amphibian eggs and *Acetabularia mediterranea. Dev. Biol.* **9:** 398–434.

Bryant, P. J., and J. H. Sang. 1968. Lethal derangements of metamorphosis and modifications of gene expression caused by juvenile hormone mimics. *Nature* **220:** 393–394.

Clegg, J. S., A. H. Warner, and F. J. Finamore. 1967. Evidence for the function of P^1,P^4 diguanosine 5′-tetraphosphate in the development of *Artemia salina. J. Biol. Chem.* **242:** 1938–1967.

Ewing, R. D., and F. J. Finamore. 1970a. Phospholipid metabolism during development of the brine shrimp *Artemia salina.* I. Incorporation of cytidine 5′-phosphate into cytidine diphosphate choline by a microsomal enzyme system. *Biochem. Biophys. Acta* **218:** 463–473.

Ewing, R. D., and F. J. Finamore. 1970b. Phospholipid metabolism during development of the brine shrimp *Artemia salina.* II. Synthesis of phosphatidylcholine by a microsomal enzyme system from nauplii. *Biochem. Biophys. Acta* **218:** 474–481.

Ewing, R. D., G. L. Peterson, and F. P. Conte. 1972. Larval salt gland of *Artemia salina* nauplii. Effects of inhibitors on survival at various salinities. *J. Comp. Physiol.* **80:** 247–254.

Hernandorena, A. 1970. Obtention de morphogenèses appendiculaires abortive et surnuméraire chez *Artemia salina* L. (Crustacé Branchiopode) par carences alimentaires de base pyrimidique et de nucléotide purique. *C.R. Séances Hébd. Acad. Sci. Paris, Sér. D* **271:** 1406–1410.

Hernandorena, A. 1972. Signification morphogénètique du besoin alimentaire en acides nucléiques chez *Artemia salina* (L.). II. Besoins en dérivés puriques. *Arch. Zool. Exp. Gen.* **113:** 489–498.

Hernandorena, A. 1974. Effects of salinity on nutritional requirements of *Artemia salina. Biol. Bull.* **146:** 238–248.

Hernandorena, A. 1975. Metabolic significance in nucleic acid metabolism and protein synthesis of dietary AMP requirement in *Artemia salina* (L.). *Biol. Bull.* **148:** 416–428.

Hernandorena, A. 1976. Adaptation métabolique à un régime lipidique, croissance et morphogenèse chez *Artemia salina* (L.). *Ann. Nutr. Alim.* **30:** 629–636.

Hernandorena, A. 1977. Action antagoniste de nucléotides purique et pyrimidique alimentaire sur la morphogenèse appendiculaire d' *Artemia salina* (L.). (Crustacé Branchiopode). *C.R. Séances Hebd. Acad. Sci. Paris, Sér. D* **284:** 1337–1339.

HERNANDORENA, A. 1979. Relationships between purine and pyrimidine dietary requirements and *Artemia salina* morphogenesis. *Comp. Biochem. Physiol.* **62B:** 7–12.

HILLMAN, R., S. J. SHAFER, AND J. H. SANG. 1973. The effects of inhibition of protein synthesis on the phenotype of abnormal abdomen. *Genet. Res.* **21:** 229–239.

PROVASOLI, L., AND A. D'AGOSTINO. 1969. Development of artificial media for *Artemia salina*. *Biol. Bull.* **136:** 434–453.

SANG, J. H., AND B. BURNET. 1963a. Physiological genetics of melanotic tumors in *Drosophila melanogaster*. I. The effect of nutrient balance on tumor penetrance in the tu^K strain. *Genetics* **48:** 235–253.

SANG, J. H., AND B. BURNET. 1963b. Environmental modification of the eyeless phenotype in *Drosophila melanogaster*. *Genetics* **48:** 1683–1699.

SANG, J. H., AND B. BURNET. 1967. Physiological genetics of melanotic tumors in *Drosophila melanogaster*. IV. Gene-environment interactions of tu bw with different third chromosome. *Genetics* **56:** 743–754.

WARNER, A. H., AND D. K. MCCLEAN. 1968. Studies on the biosynthesis and role of diguanosine tetraphosphate during growth and development of *Artemia salina*. *Dev. Biol.* **18:** 278–293.

Reference: *Biol. Bull.*, **160:** 272–279. (April, 1981)

L2

RESPIRATORY ADAPTATIONS OF THREE SPECIES OF *UPOGEBIA* (THALASSINIDEA, CRUSTACEA) WITH SPECIAL REFERENCE TO LOW TIDE PERIODS

BURKE HILL*

Zoology Department, Rhodes University, Grahamstown 6140, South Africa

Abstract

At simulated low tide, *Upogebia africana, U. affinis*, and *U. capensis* moved to the air-water interfaces of their burrows and took up positions in which the cephalothorax was out of the water. The posterior end of the gill chamber dipped into the water and continuing scaphognathite beat pulled water up through the gill chamber. This behavior was induced in *U. africana* by exposure to low oxygen tension (pO_2) water. The median survival time of *U. africana* was 18 h in anoxic conditions and 72 h in air. *U. africana* has a low metabolic rate that may be an adaptation to hypoxic conditions. The blood pO_2 decreased rapidly when the shrimps were exposed to hypoxia and reached an equilibrium level after about 5 h. The pO_2 of blood of *U. africana* in air was approximately half that of shrimps in normoxic water.

Introduction

Thalassinid shrimps occupy burrows in the soft substrates typical of many intertidal and shallow subtidal areas throughout the world (Macginitie 1930, 1934). These substrates frequently are hypoxic. In addition, those species living in intertidal areas cannot irrigate their burrows each day during periods of low tide. A number of behavioral and physiological respiratory adaptations to these hypoxic habitats have been described. Such adaptations in species of *Callianassa* include reduced metabolism at low oxygen tensions (Torres *et al.*, 1977), high affinity hemocyanin (Miller and Van Holde, 1974), tolerance to anoxia (Thompson and Pritchard, 1969), and burrow ventilation behavior related to oxygen tension (Farley and Case, 1968). Less information is available on respiratory adaptations of species of *Upogebia*, but they appear to be similar to those of *Callianassa*. Thompson and Pritchard (1969), for example, report that *Upogebia pugettensis* reduces its metabolic rates in low oxygen tensions and tolerates considerable anoxia.

The present study describes behavior under low tide conditions in burrows in three species of *Upogebia: U. africana*, a common inhabitant of intertidal mud banks in estuaries in southern Africa; *U. affinis*, which occupies a similar habitat on the Atlantic coast of North America (Pearse, 1945); and *U. capensis*, a marine species found on the southern and western coast of southern Africa, more commonly subtidally than intertidally (Barnard, 1950). Metabolic rates, tolerance to anoxia, and oxygen tension of the blood under hypoxia also were measured in *U. africana.* //

Received 8 September 1980, accepted 27 December 1980. .

Abbreviations: Standard deviation, SD; standard error, SE; oxygen tension, pO_2; blood oxygen tension, p_iO_2; oxygen tension in surrounding water, p_eO_2.

* Present Address: Queensland Fisheries Service, P. O. Box 344, Fortitude Valley, Queensland, 4006, Australia.

MATERIALS AND METHODS

Source of material

All *Upogebia africana* specimens used in this study were collected from mud banks in the Kowie estuary at Port Alfred, South Africa. *U. capensis* specimens were obtained from the rocky shore around Port Alfred. Observations were made at Rhodes University, Grahamstown. *U. affinis* specimens were collected from intertidal mud flats at Beaufort, North Carolina, and behavioral observations made at Duke University.

Behavior at low tide

Observations were made on 10 specimens of *U. africana* kept in a glass-sided aquarium containing a 150-mm-deep layer of estuarine mud into which shrimp burrowed. Some sections of their burrows adjoined the sides of the aquarium, enabling observations of their behavior. Low tide was simulated by withdrawing all the overlying water for 6 h. Observations were made on 10 specimens of each species kept individually in U-shaped glass "burrows" with an internal diameter of 10 mm and a 20-mm-diameter enlargment at the base, where the shrimp could turn. The "burrows," with vertical height 300 mm, were filled to within 50 mm of the top with seawater (34‰). A 10–15 mm carapace–length shrimp was introduced and its behavior observed for 4–6 h. The oxygen tension of water around *U. africana* during low tide in glass burrows was determined from water samples withdrawn into a syringe and injected into a Radiometer BMS3 analyzer. The water bath of the analyzer was maintained at 20°C, the water temperature in the simulated burrows.

Behavioral response to low oxygen tensions

The effect of low oxygen tensions on *U. africana* behavior was determined on shrimp kept individually in a flowing-water system involving a U-shaped "burrow" as described above, except that 50 mm from its upper ends, the burrow was fitted with small side arms through which water (34‰, 18°C) flowed at about 3 $\text{l} \cdot \text{h}^{-1}$ from a supply reservoir. The oxygen tension of water in the system could be lowered by bubbling nitrogen through the water in the supply reservoir. An oxygen electrode was used to measure continuously the oxygen tension of water passing through the "burrow." A 10–15 mm carapace-length shrimp (1.5–2 g) was put into the burrow and allowed to settle down for 1 h at a pO_2 of 140–148 mm Hg. The oxygen tension was then slowly reduced to about 5 mm Hg over approximately 1 h. The pO_2 was then gradually increased by aerating water in the reservoir. The shrimp's behavior was noted at various oxygen tensions. The procedure was repeated on 10 animals. Control experiments were carried out using shrimps kept in the system in aerated seawater.

Tolerance to anoxia

Tolerance of *U. africana* to anoxia was determined in circular glass dishes 250 mm in diameter containing about 5 l (depth approximately 100 mm) of seawater (34‰, 18°C). Nitrogen was bubbled through the water by means of inlet and outlet tubes in the glass lids. The outlet tube was fitted with a water trap. When the oxygen tension of samples of the water was below 0.5 mm Hg, 10 shrimps were put into each of four dishes. A fifth dish containing aerated water and 10 shrimps was used as a control. The number of shrimp alive was counted at irregular intervals for 24 h. Oxygen tension in the experimental dishes remained below 0.5 mm Hg. Water was not replaced during the experiment.

Survival in air

Two batches each of 20 specimens of *U. africana* were put into covered 250-mm-diameter glass dishes. The dishes contained a 10-mm-deep layer of freshwater, but a perforated plastic floor prevented the shrimp from contacting the water. Water-saturated air was pumped through the dishes at 400 $ml \cdot h^{-1}$. Ten specimens of *U. africana* were kept in a dish of aerated seawater as a control. The experiment was run at 20°C. Survival of the shrimps was checked at irregular intervals. The blood pO_2 of an additional 10 specimens of *U. africana* kept in water-saturated air was measured after 24 h exposure.

Oxygen consumption

Oxygen consumption of *U. africana* at controlled temperatures was measured in a flowing-water respirometer. A four-channel peristaltic pump transferred filtered seawater (34‰) from a 25 l glass reservoir to four respirometer chambers. Water leaving the chambers could be either diverted to waste or passed over an oxygen electrode (Radiometer). A bypass system was used to measure the pO_2 of water entering the chambers. Experimental animals were kept for 24 to 72 h in clean tanks containing aerated seawater. They were then put individually into a respirometer chamber and allowed to acclimiate for 2 h. During this period the flow was adjusted so that the pO_2 of effluent water from chambers was about 85% of that of influent water. Influent and effluent oxygen tensions, and water flow were measured for three 10-min periods for each animal. At the end of the experiment the animals were removed, their surface moisture was dried off with a paper towel, and they were weighed to the nearest 0.01 g. Consumption at 20 ± 0.5°C was determined on 60 shrimps in the range 0.2–3.5 g. Shrimp were collected in summer and the experimental temperature corresponded to the mean habitat temperature at that time (Hill and Allanson, 1971). Since activity affects oxygen consumption, measurements used in this paper are restricted to those taken when the shrimp exhibited slight activity—slow walking movements and slow pleopod beat. Vigorous activity involving rapid pleopod beat usually continued only for short periods.

Oxygen tension of blood

The rate at which the oxygen tension of blood dropped after exposure to low oxygen tension was determined on four batches each of 10 specimens of *U. africana*. The shrimps were kept for 24 h at a pO_2 of 140–148 mm Hg and a temperature of 20°C, in the dishes used for the anoxia tolerance experiments described above. The water was then replaced by water equilibrated to a pO_2 of 38 mm Hg and the tension maintained by vigorous bubbling with a gas mixture at the same tension. Blood was sampled from one batch after 1 h, a second after 3 h, a third after 13 h, and the fourth after 22 h. A control batch was kept in aerated seawater.

In a second experiment the relationship between blood oxygen tension (p_iO_2) and surrounding water tension (p_eO_2) was determined on four batches of 10 shrimp each. Each batch was kept at a different oxygen tension (112, 71, 38, or 19 mm Hg) for 6 h before blood samples were withdrawn. In all cases blood samples were extracted from the pericardial space by means of a syringe, injected into a Radiometer BMS3 blood gas analyzer, and the pO_2 measured. The analyzer water bath was maintained at 20°C.

Results

Behavior at simulated low tide

About 1 hr after a simulated low tide began, *U. africana* in either glass burrows or mud burrows in an aquarium moved slowly up the burrow and approached the air-water interface. When its antennae reached the surface, the shrimp stopped, and in some cases moved down the burrow, before recommencing upward movement. Intermittent strong pleopod beat lowered the water level in the arm of the burrow containing the shrimp. The shrimp gradually moved higher in the burrow until its cephalothorax was out of the water and its abdomen was still submerged (Fig. 1). In this position the hindmost edge of the branchial chamber was underwater and the chamber remained full of water. The scaphognathites continued beating and a stream of water flowed from the anterior end of the gill chamber. This water ran down the ventral and ventrolateral surfaces of the shrimp and into the burrow water. At irregular intervals the animals retreated briefly beneath the surface. If an emerged shrimp was disturbed it retreated underwater but re-emerged within a few minutes. The mean pO_2 of water surrounding *U. africana* half emerged in this static system was 54.3 mm Hg (standard deviation, SD, 7.37, n = 10).

During simulated low tide, *U. capensis* gradually approached the surface, eventually emerging so that the water level was at the thorax-abdomen junction or even

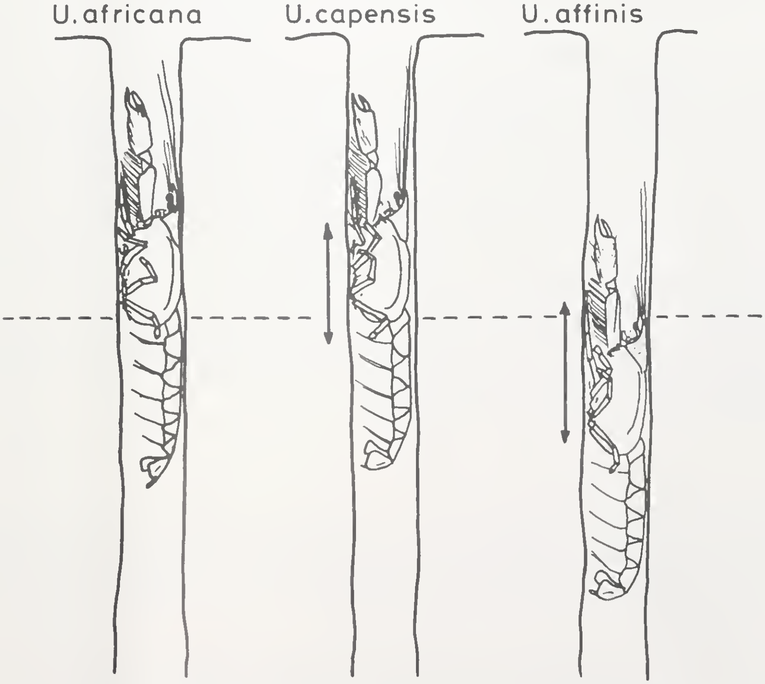


FIGURE 1. Positions assumed by three species of *Upogebia* at low tide. Dashed line indicates equilibrium level of burrow water. Vertical arrows indicate water movement induced by shrimp.

halfway down the abdomen. Strong pleopod beat then oscillated water in the burrow so that it washed up and down over the posterior end of the branchial chamber (Fig. 1). The caudal fan occasionally made a strong scooping movement which lifted water over the cephalothorax. The scaphognathite beat continued, maintaining a stream of water through the gill chamber.

U. affinis also slowly approached the surface, and made several rapid pleopod beats before reaching it. When these beats stopped, the caudal fan pushed water forward. The shrimp repeated this several times, occasionally walking upwards a short distance on the pleopod beat. When the shrimp reached a level at which the pleopod beat pulled the water level down to the tip of the rostrum, the pleopod beat increased, pulling the water further down. The animal gradually moved higher until its rostrum was approximately at the undisturbed water level. Pleopod beat now drew water down until its surface was approximately level with the junction of thorax and abdomen (Fig. 1). The water was held at this level by the pleopod beat and by a ventral flexing of the sixth abdominal segment, which caused the caudal fan to act as a barrier. After a few seconds to several minutes, the shrimp stopped its pleopod beat and relaxed its caudal fan, suddenly releasing the water. This caused the water level to rise rapidly up over the cephalothorax. The extent to which water was pulled down varied; the maximum was halfway down the abdomen. One specimen never exposed its body above water.

Thus, at simulated low tide, all three species took up a position in which the anterior half of the body was in air but in which the scaphognathites still pumped water through the branchial chamber. The shrimps appeared to avoid drying of the cephalothorax by periodic wetting, although by a different mechanism in each species.

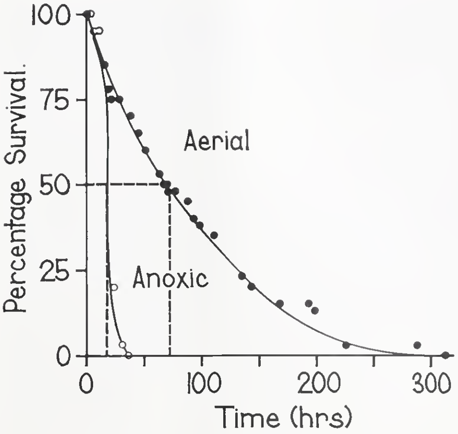
Effect of low oxygen tensions

U. africana exposed to low pO_2 in a glass burrow showed the same behavior as was observed under simulated low tide conditions. Various stages of the behavior pattern could be related to particular ranges of pO_2. Shrimp remained submerged when aerated water at a pO_2 of 145–150 mm Hg was passed through the burrow for up to 15 h. If the pO_2 was lowered below 36 Hg, activity decreased, the pleopod beat slowed, and the gills were frequently probed by the fifth pereiopods. The shrimps emerged when the pO_2 dropped below 11 mm Hg. When the pO_2 was raised above 25 mm Hg, they descended and remained submerged but were not very active. The pO_2 at which shrimp emerged in this flowing water system was lower than that required in the static system (see above). The difference may be due to a buildup of metabolites in the latter.

Tolerance to anoxia and survival in air

Because of the similar results of three replicate experiments on survival of *U. africana* in anoxic water, the results were grouped and an overall percentage survival was calculated (Fig. 2). Nearly all the shrimp survived anoxia for about 12 h, but died with longer exposure. Median survival time was 18 h. No shrimp died in the control experiment.

Fifty percent of specimens exposed to air died within 72 h (Fig. 2). At this time, 90% of control animals were still alive. The mean pO_2 of *U. africana* blood after 24 h in air was 37 mm Hg (standard error, SE, of mean 2.9).



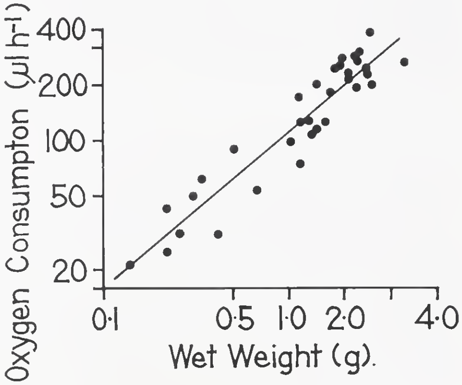


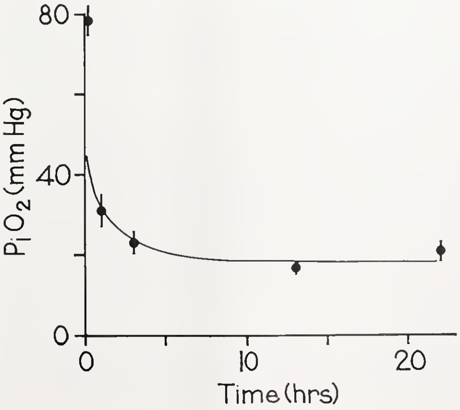
FIGURE 2. (left) Survival of *U. africana* specimens in anoxic (open circles) and aerial (solid circles) conditions. In each case 100% = 40 shrimps. Lines fitted by eye. Median time of survival in anoxia 18 h, in air 72 h.

FIGURE 3. (right) Oxygen consumption of *U. africana* specimens of various sizes at 20 ± 0.5°C. Line fitted by regression analysis ($r^2 = 0.8823$, $n = 60$).

Oxygen consumption and oxygen tension of the blood

The relationship between wet weight of *U. africana* and oxygen consumption ($\mu l \cdot h^{-1}$) at 20°C is shown in Figure 3, and is described by the equation log oxygen consumption ($\mu l \cdot h^{-1}$) = log 112.2 + 0.8446 log wet weight (g). The 95% confidence interval for b (slope) is 0.7321–0.9571.

The oxygen tension of *U. africana* blood declined rapidly when the shrimp were exposed to a pO_2 of 38 mm Hg (Fig. 4). A p_iO_2 of around 20 mm Hg was reached after 5 h and maintained for at least 22 h. In normoxic water (pO_2 140–150 mm



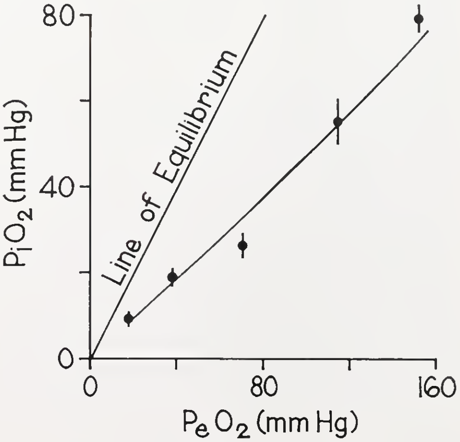


FIGURE 4. (left) Oxygen tension of the blood of *U. africana* specimens exposed to a pO_2 of 38 mm Hg (n = 10 at each point, vertical lines indicate 2 SE).

FIGURE 5. (right) Partial pressure of oxygen in the blood of *U. africana* specimens at various environmental oxygen tensions (n = 10 at each point, vertical lines indicate 2 SE).

Hg), *U. africana* had a p_iO_2 of around 80 mm Hg. In lower oxygen tensions the difference between p_iO_2 and p_eO_2 decreased but the blood oxygen tension was always approximately half that of the surrounding water (Fig. 5).

DISCUSSION

Several measurements of oxygen consumption by thalassinids are now available. Thompson and Pritchard (1969) reported rates of 30 and 60 $\mu l \cdot g^{-1} \cdot h^{-1}$ at 10°C for 3–8 g specimens of *Callianassa californiensis* and *U. pugettensis*, respectively. Felder (1979) found similar rates (50–60 $\mu l \cdot g^{-1} \cdot h^{-1}$) for 3.5–5 g specimens of *C. jamaicense* at 25°C. Torres *et al.* (1977) reported a low rate of 14 $\mu l \cdot g^{-1} \cdot h^{-1}$ at 14°C for 10–15 g specimens of *C. californiensis*, and Miller et al. (1976) measured 30–40 $\mu l \cdot g^{-1} \cdot h^{-1}$ for 6–7 g specimens of the same species. Comparison of rates is difficult because size and temperature affect oxygen consumption. *U. africana* is a smaller species than the thalassinids used in the above studies. The average adult *U. africana* obtainable (3 g) used around 100 $\mu l \cdot g^{-1} \cdot h^{-1}$ at 20°C. This rate is comparable to those reported for similar sized thalassinids and confirm the general trend noted by previous authors that thalassinids have low metabolic rates as compared to other crustacea. Thompson and Pritchard (1969) and Felder (1979) considered that these low metabolic rates reflect an adaptation to a hypoxic habitat.

The size of *U. africana* burrows varies widely, but personal observations indicate that on average a 2 g shrimp occupies a burrow with a volume around 80 ml. At 20°C this volume of seawater contains about 400 μl O_2. A 2 g *U. africana* would consume approximately 200 μl $O_2 \cdot h^{-1}$. Thus the oxygen in the burrow water is insufficient to supply the occupant over a low tide. The blood pO_2 of *U. africana* drops rapidly when the shrimps are exposed to low oxygen (Fig. 4). According to Miller *et al.* (1976), *C. californiensis* blood has a low oxygen content. Thus twice each day at low tide, thalassinids have to use alternative mechanisms to cope with hypoxic conditions. The short survival of *U. africana* in anoxic conditions (Fig. 2) indicates that, in this species, anaerobic metabolism is not well developed. All species of *Upogebia* so far tested appear to have a low tolerance to hypoxia. Thompson and Pritchard (1969) showed that *U. pugettensis* was less tolerant of anoxic stress than was *C. californiensis*. Part of the mechanism of this difference was shown by Miller *et al.* (1977) to be due to physiological properties of the blood, in that hemocyanin of *C. californiensis* has a higher affinity for oxygen (p_{50} 4 mm Hg) than that of *U. pugettensis* (p_{50} 11.5 mm Hg).

When exposed to hypoxic conditions in a simulated burrow, specimens of all three species of *Upogebia* tested in the present study showed a characteristic behavior that involved moving to the air-water interface. Farley and Case (1968) reported that *C. californiensis* appears to move towards the openings of its burrows at low tide. Felder (1979) found specimens of *C. jamaicense* in the substrate well above the water table. He suggested that since *C. jamaicense* can survive and respire in water-saturated air, occupancy of exposed upper portions of burrows seems a plausible alternative to long-term anoxia.

U. africana appears to have some ability to respire in air, since it survived better out of water than in anoxic conditions. Nevertheless, the shrimp's oxygen uptake in air was not highly efficient, since in air its p_iO_2 (37 mm Hg) was half that in water (78 mm Hg). Mortality in air was probably not due to lack of oxygen but to a multiplicity of factors, including a buildup of metabolic products. The behavior of the three species of *Upogebia* at low tide did not involve the shrimps leaving the water completely. They took up a position in which water could be pumped through the gill chamber despite the fact that the cephalothorax was exposed. This water, being at the air-water interface, could be expected to have a higher oxygen content than that lower down in the burrow. Water trickling back down over the ventral surface of the shrimp after leaving the gill chamber may be partly reoxygenated. A similar mechanism has been described in the grapsid crab *Sesarma catenata* by Alexander and Ewer (1969). In this crab water can be stored in the gill chamber, whereas in *Upogebia*, continued irrigation of the gills relies upon the shrimp's maintaining contact with burrow water.

Callianassa species live in temporary burrows in permeable sandy substrates. They are continually exposed to low levels of oxygen and require a well developed tolerance to hypoxia. A similar high resistance to anoxic stress is not necessary in *Upogebia* because the shrimps live in a burrow which is sealed from interstitial water (Thompson and Pritchard, 1969) and have a specialized respiratory behavior at low tide.

ACKNOWLEDGMENTS

The financial assistance of the South African C.S.I.R. is gratefully acknowledged.

LITERATURE CITED

ALEXANDER, S. J., AND D. W. EWER. 1969. A comparative study of some aspects of the biology and ecology of *Sesarma catenta* and *Cyclograpsus punctatus*. *Zool. Afr.* **4:** 1–35.

BARNARD, K. H. 1950. Descriptive catalogue of South African Decapod Crustacea. *Ann. S. Afr. Mus.* **38:** 1–837.

FARLEY, R. D., AND J. F. CASE. 1968. Perception of external oxygen by the burrowing shrimp *Callianassa californiensis* Dana and *C. affinis* Dana. *Biol. Bull.* **134:** 261–265.

FELDER, D. L. 1979. Respiratory adaptations of the estuarine mud shrimp *Callianassa jamaicense*. *Biol. Bull.* **157:** 125–137.

HILL, B. J., AND B. R. ALLANSON. 1971. Temperature tolerance of the estuarine prawn *Upogebia africana*. *Mar. Biol.* **11:** 337–343.

MACGINITIE, G. E. 1930. The natural history of the mud shrimp *Upogebia pugettensis* Dana. *Ann. Mag. Nat. Hist. Ser. 10.* **6:** 36–44.

MACGINTIE, G. E. 1934. The natural history of *Callianassa californiensis* Dana. *Am. Midl. Nat.* **15:** 166–177.

MILLER, K. I., AND K. E. VAN HOLDE. 1974. Oxygen binding by *Callianassa californiensis* hemocyanin. *Biochemistry* **13:** 1668–1674.

MILLER, K. I., A. W. PRITCHARD, AND P. S. RUTLEDGE. 1976. Respiratory regulation and the role of blood in the burrowing shrimp *Callianassa californiensis* (Decapoda: Thalassinidea). *Mar. Biol.* **36:** 233–242.

MILLER, K. E., N. W. ELDRED, F. ARISAKA, AND K. E. VAN HOLDE. 1977. Structure and function of hemocyanin for thalassinid shrimp. *J. Comp. Physiol.* **114:** 171–184.

PEARSE, A. S. 1945. Ecology of *Upogebia affinis* (Say). *Ecology* **26:** 303–305.

PRITCHARD, A. W., AND S. EDDY. 1979. Lactate formation in *Callianassa californiensis* and *Upogebia pugettensis* (Crustacea: Thalassinidea). *Mar. Biol.* **50:** 249–253.

THOMPSON, R. K., AND A. W. PRITCHARD. 1969. Respiratory adaptations of two burrowing crustaceans, *Callianassa californiensis* and *Upogebia pugettensis* (Decapoda, Thalassinidea). *Biol. Bull.* **136:** 274–287.

TORRES, J. J., D. L. GLUCK, AND J. J. CHILDRESS. 1977. Activity and physiological significance of the pleopods in the respiration of *Callianassa californiensis* (Dana) (Crustacea: Thalassinidea). *Biol. Bull.* **152:** 134–146.

Reference: *Biol. Bull.*, **160:** 280–291. (April, 1981)

THE INFLUENCE OF TEMPERATURE ON THE KINETICS OF ALLOGRAFT REACTIONS IN A TROPICAL SPONGE AND A REEF CORAL*

I. S. JOHNSTON,[1] P. L. JOKIEL,[2] C. H. BIGGER,[1] AND W. H. HILDEMANN[1]

[1]*Immunogenetics Group, School of Medicine and Dental Research Institute, University of California, Los Angeles, California, 90024, USA; and*
[2]*Hawaii Institute of Marine Biology, Kaneohe, Hawaii 96744, USA*

ABSTRACT

Many tropical sponges and reef-building corals demonstrate highly discriminating transplantation immunity when grafted with allogeneic tissue. The speed of rejection changes seasonally, and therefore the role of temperature was investigated. Replicate parabiotic allografts of a Hawaiian sponge, *Callyspongia diffusa*, and a reef-coral, *Montipora verrucosa*, were exposed to three different temperature regimens: approximately 23°, 25°, and 27°C. The primary allograft reactions were extremely temperature dependent in both species; the median reaction times (MRT) for the first-set grafts at summer temperatures (27°C) were approximately half the winter temperature (23°C) MRTs. Significantly accelerated second-set reactions in the coral were similarly affected by temperature; however, maximally accelerated second-set reactions in the sponge were similar at all three temperatures. The primary mean reaction times for replicate grafts varied significantly among the individual genotypic combinations brought into parabiosis. Furthermore, different genotypic combinations responded to temperature changes with qualitatively distinctive reaction trends as well as different reaction times. Parabiotic allografts mimic intraspecific interactions that are a consequence of natural contact through growth of sedentary marine animals. The influence of temperature on the outcome of intraspecific competition suggests that temperature fluctuations could maintain high levels of genetic polymorphism within individual populations of sponges and corals.

INTRODUCTION

The phenomena of self versus not-self recognition and of immunocompetence among marine invertebrates are of broad interdisciplinary interest. They are relevant to considerations of: (1) ecological competition, *e.g.* intraspecific and interspecific competition for space among sedentary animals (Connell, 1976; Jackson and Buss, 1975; Bigger, 1980); (2) population genetics, *e.g.* the benefits and maintenance of genetic polymorphism and strategies for sexual versus asexual reproduction (Shick and Lamb, 1977); (3) pathogenesis and defense against pathogenic organisms; (4) symbiosis, *e.g.* the specificity and stability of algal-invertebrate symbioses (Trench, 1979), and (5) immunology, *e.g.* phylogenetic trends in immune systems (Manning and Turner, 1976).

Intimate and prolonged tissue contact between conspecific but genotypically

Received 17 October 1980; accepted 23 December 1980.
Abbreviations: MRT, median reaction time; COCO, KYC, collection sites.
* Contribution No. 594 of the Hawaii Institute of Marine Biology.

nonidentical individuals, *i.e.* allogeneic individuals, in certain reef corals (Raison *et al.*, 1976; Hildemann, Raison, Cheung, *et al.*, 1977) and sponges (Hildemann *et al.*, 1979; Van de Vyver, 1980) can generate immune-type transplantation rejection. Allogeneic graft rejection results in zones of tissue death occurring unilaterally or more commonly bilaterally at the contact interface. These sponge and coral reactions are characterized by discriminating allogeneic recognition and subsequent cytotoxicity. At least some species display accelerated responses towards subsequent contact with the same allogeneic individuals, in a way that indicates immunological memory (Hildemann *et al.*, 1979; Hildemann, Bigger, Jokiel, *et al.* 1980; Hildemann, Jokiel, *et al.* 1980; Evans *et al.*, 1980). We believe that these properties of sponge and coral allograft reactions fulfill minimal criteria for their full identification with other immunological phenomena (Hildemann, Bigger, Jokiel, *et al.*, 1980).

In higher animals, allo-transplantation reactions are usually induced artificially by surgical means, though maternal-fetal incompatibilities occur naturally in many mammals. Such allogeneic incompatibilities probably express surveillance mechanisms that normally detect and destroy antigenically transformed, potentially cancerous cells, or pathogenic microorganisms. Among sedentary benthic marine animals, allogeneic tissue contacts or grafts are a natural result of vegetative growth, especially in encrusting organisms. When contact occurs during coral and sponge growth, alloreactive soft tissues do not fuse and a vacant zone of variable width is created between the colonies (Hildemann, Raison, and Hull, 1977; Curtis, 1979). This outward expression may at first sight appear equivalent to the end results of certain xenogeneic, or interspecific, interactions among animals competing for the same space or feeding upon one another. However, the underlying mechanisms that achieve and maintain separation may be quite different (see Lang, 1973; Hildemann, Raison, Cheung, *et al.*, 1977).

In ectothermic invertebrates it is not surprising to find physiological responses that depend quantitatively and even qualitatively on temperature. Much work has been done with corals to determine the effect of temperature on growth and mortality (Jokiel and Coles, 1977; Houck *et al.*, 1977), calcification (Clausen and Roth, 1975), photosynthesis and respiration (Coles and Jokiel, 1977), and reproduction (Jokiel and Guinther, 1978). Synergistic effects of temperature with light and salinity have also been investigated (Coles and Jokiel, 1978). However, little is known about general temperature effects in sponges (Sarà and Vacelet, 1973) other than an effect on pumping rates (Reiswig, 1971). Nothing was known about how temperature changes might affect allograft reactions in sponges and corals until we observed that they rejected allografts more rapidly at higher ambient sea water temperatures during the summer (Hildemann *et al.*, 1979). Other workers have now also become alert to the possibility that ambient temperature changes may significantly affect alloincompatibilities in sponges (Evans *et al.*, 1980; Van de Vyver, 1980). The close dependence of diverse immune responses on environmental temperature has long been known in ectothermic fishes, amphibians, and reptiles (Hildemann, 1962). The published accounts of sponge grafting experiments carried out in temperate vs. tropical areas need explanation, because some reports from cooler areas have not noted alloincompatibility and/or concomitant cytotoxicity (Moscona, 1968; Paris, 1961). Here, we report the substantial effect of temperature on alloreactivity under controlled laboratory conditions in the tropical sponge *Callyspongia diffusa* and the reef-coral *Montipora verrucosa* over the normal Hawaiian range of 22–28°C.

MATERIALS AND METHODS

We conducted our experiments at the Hawaii Institute of Marine Biology (Coconut Island, Oahu) in fiberglass aquaria (1 × 1 × 0.5 m) in full natural sunlight shaded to 30% of incident illumination with black nylon window screen. The aquaria were constantly aerated and supplied with non-recirculating unfiltered sea water pumped from Kaneohe Bay at a rate sufficient to flush them in less than 1 h. Each of three identical arrays of aquaria were subject to different temperature regimens: approximately 23°C, 25°C, and 27°C. The 25°C experiment was simply exposure to the ambient temperature of the incoming water. In the 23°C experiment, the incoming water was chilled by 2°C below ambient while in the 27°C experiment, the incoming water was heated by 2°C. Only inert materials contacted the sea water; pump and piping surfaces were of non-toxic plastic; heat exchange surfaces were of titanium and glass. Temperatures were measured daily with a mercury thermometer calibrated to 0.1°C. The aquarium temperatures followed the slight (less than 1°C) natural diurnal temperature cycle observable on the reefs, with afternoon temperatures slightly warmer than those observed in the morning. Table I shows ambient temperatures, and the precision with which the cooler and warmer treatments were controlled. The range of sea water temperatures over which these experiments were performed is well within the yearly range of monthly means of 22–28°C for Kaneohe Bay (*cf.* Jokiel and Coles, 1977, Fig. 3). A seasonal warming trend in ambient temperatures was only marginally evident during the experments.

The corals and sponges used in the experiments were collected from two widely separated reefs in Kaneohe Bay, to prevent comparisons of genetically similar or identical animals derived vegetatively from the same parent colony. One site was a shallow patch reef north of Coconut Island (henceforth referred to as COCO). The second reef was located across the Bay, some 3 km to the south near the Kaneohe Yacht Club (henceforth referred to as KYC). The two sites are separated by a deep (10 m) lagoon with a sand or mud bottom unsuitable for the species studied. Any cross colonization must therefore be through dispersal of motile larval forms.

TABLE I

Ambient temperatures and temperature control during the course of Callyspongia diiffusa *and* Montipora verrucosa *grafting experiments.*

	Mean ambient temperature (°C ± SD) 25°C Treatment		Mean of temperature differences from ambient (°C ± SD) (simultaneous measurements made at any time of day between 07:15 and 17:30 h)	
	Morning 07:30–10:30 h	Afternoon 13:00–16:00 h	23°C chilled treatment	27°C heated treatment
Data collected over the duration of the sponge experiment: 19 April–9 May 1979	25.1 ± 0.3	25.8 ± 0.3	−2.3 ± 0.5	+2.1 ± 0.2
Data collected over the duration of the coral experiment: 23 April–8 July 1979	25.3 ± 0.4	25.9 ± 0.5	−2.3 ± 0.6	+1.9 ± 0.4

The two species studied occur on reefs at both sites. *Callyspongia diffusa* is a large, violet, branched sponge found on shallow subtidal reef flats and not in deeper water. The reef coral *Montipora verrucosa* occurs in such shallow areas, but is more abundant along the deeper portions of the reef face. This species is one of the most polymorphic species of coral known, assuming encrusting, branching, massive, or foliacious (plate-like) growth forms (Vaughan, 1907).

Eight large colonies of *C. diffusa* were obtained from each site. The COCO colonies were numbered C1–C8 and the colonies from KYC C13–C20. Six branch tips, approximately 6–8 cm long, were excised from each parent colony. Branch tips from different colonies were grafted together by immobilizing them in contact with each other, as previously described by Hildemann *et al.* (1979). Each piece of sponge from colony C1 was grafted with a piece from colony C13, and so on with the combinations C2–C14 through C8–C20. The grafts interfaced by at least 2–3 cm of linear contact between the presumed allogeneic sponge pairs. The grafts did not involve cut surfaces of sponge tissue, but rather intimate physical contact of intact pinacoderm surfaces, mimicing natural contact grafts. We have called these "parabiosis grafts". Each replicated set of graph pairs (n = 6) is equivalent to replicated grafts between two different, highly inbred populations of laboratory animals. The six replicates of each interclonal combination were equally divided among each of the three temperature regimens.

Large colonies of the flat-plate growth form of *M. verrucosa* were also harvested from each site. Using heavy bone shears, we cut each colony into six genetically identical plates, each with 6–10 cm of intact growing edge from the original colony. Parabiosis grafts were set up with at least 3–4 cm of intimate skeletal and soft tissue contact interface along undamaged growing edges (after Hildemann, Raison, Cheung, *et al.*, 1977). The numbering of clones, total number of allografts, and assignment of pairs to the three temperature regimens was the same as described for *C. diffusa*, with the prefix M rather than C.

We observed the daily progress of alloreactions in both sponge and coral grafts until grafts were definitively rejected, and the time to this point was recorded as the rejection time (days). We chose an objectively convenient end point, i.e. when the soft tissues were killed back to 1 mm on either or both sides of a graft interface. In both species, allogeneic killing reveals naked underlying skeletal materials: a brown spongin fiber network in *C. diffusa* (Fig. 1; *cf.* Hildemann *et al.*, 1979, Fig. 1B), and the white calcium-carbonate corallum in *M. verrucosa* (Fig. 2; *cf.* Hildemann, Raison, Cheung, *et al.*, 1977, Fig. 2). Using suitable magnification aids, we measured the extent of tissue killing with an accuracy of 0.1 mm. Following definitive reactions in all "first-set" grafts in a given temperature treatment, these graft pairs were separated and immediately regrafted together at new interfaces, i.e. as "second-set" grafts. The progress of each second-set graft was similarly recorded until definitive rejection.

Since second-set graftings could only be made when the primary reactions were complete, second sets could not be started simultaneously, as were the first-set grafts. Therefore, second-set replicates in each temperature treatment were not necessarily concurrent. This was not a major concern because the temperature regimens were stable.

RESULTS

Tables II and III give first- and second-set mean reaction times for the replicated grafts in each temperature treatment. The differences among reaction times for

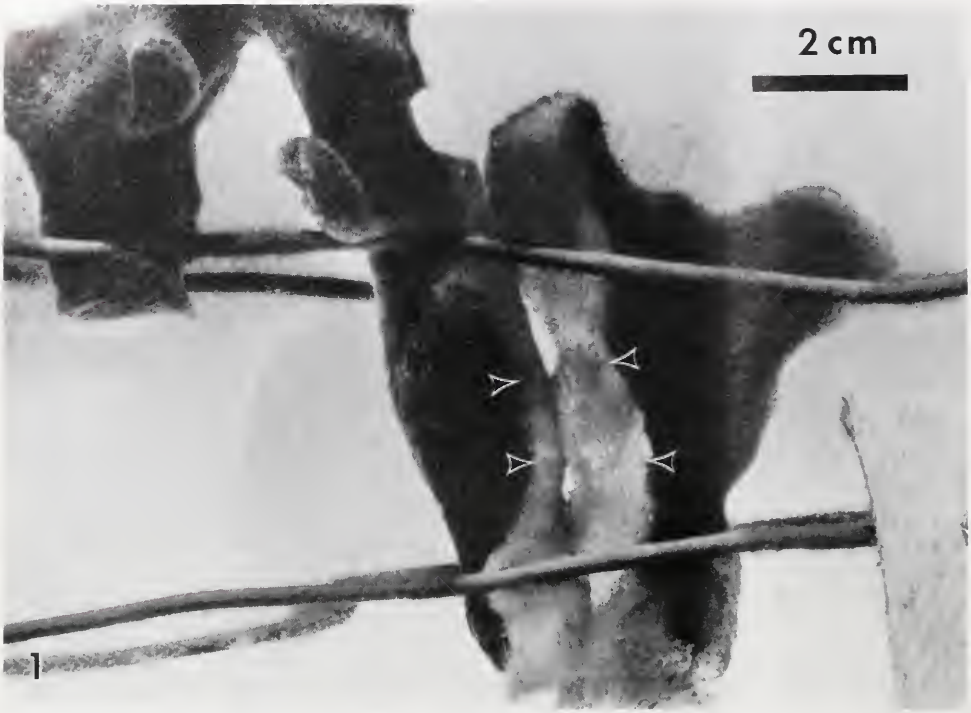


FIGURE 1. Allogeneic killing in an incompatible parabiotic graft of the sponge, *Callyspongia diffusa*. A bilateral cytotoxic reaction has exposed the spongin fiber skeletal framework at the contact interface (between apposing arrow heads).

identical interclonal grafts at the same temperature were small, as previously shown for *C. diffusa* (Johnston and Hildemann, in press). In the present experiment, 7 of 24 replicated pairs of first-set *M. verrucosa* grafts had identical reaction times while 10 more had reaction times within 1–4 days of each other. On the other hand, reaction times for grafts assembled from different interclonal combinations differed considerably: for example, compare mean reaction times between the sponge first-set combinations C3–C15 and C4–C16, and between the coral first-set combinations M4–M16 and M8–M20. Therefore, at a given temperature the cytotoxic reactivity provoked by allogeneic cell-surface contact among random pairs of sponges or corals was reflected in a continuum of reaction times: some were faster and some were slower depending on the genotypic combination of the individual graft pairs. Certain pairings consistently reacted rapidly at all temperatures while others reacted more slowly.

Since the same combinations were replicated at each temperature, we made paired comparisons to look for an overall temperature effect in spite of the variation introduced by different genotypic graft sources. Table IV summarizes the results of paired-comparisons *t* tests performed on the reaction-time differences for each of the two temperature increments in both first- and second-set grafts, and also on the first-set to second-set differences within each temperature treatment. For both sponges and corals, first-set reaction times decreased significantly over each temperature increment, *i.e.* between 23°C and 25°C, and again between 25°C and 27°C. By contrast, over the same temperature increments neither species showed

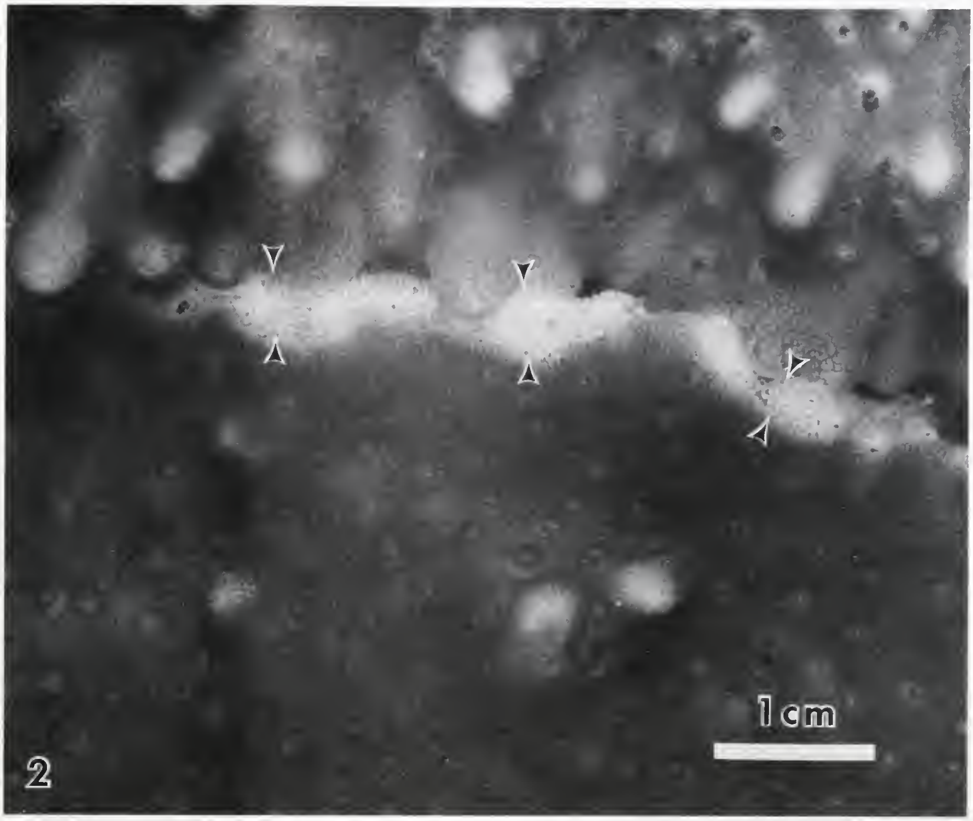


FIGURE 2. Allogeneic killing in an incompatible parabiotic graft of the reef-coral, *Montipora verrucosa*. In the lower individual a unilateral cytotoxic reaction at the contact interface has exposed the underlying calcium carbonate corallum (between apposing arrow heads).

a significant temperature effect on second-set reaction times. In both animals at a given temperature, second-set reactions were significantly faster than first-set reactions, except for coral allografts at the warmest (27°C) temperature. Two-way analysis of variance for first-set reaction times in both animals showed significant variation resulting from (i) temperature, (ii) individual graft pairing, *i.e.* genotypic combination, and (iii) interaction between temperature effect and genotypic combination. This latter interaction is illustrated by a comparison between the trends in first-set mean reaction times over the three temperatures in coral combinations M1–M13 and M6–M18.

Graphic plots of median reaction times (derived from log-normal transformations of the data from the eight different genetic combinations tested for each animal) versus experimental temperature means (Fig. 3) illustrate the general trends in the effect of temperature on allograft reactions. Except for the sponges' strikingly accelerated second-set reactions, which were similar at all three temperatures, the grafts generally were rejected faster with increasing temperature. In other words, the sharply heightened immune reactions against repeat sponge grafts were much less affected by temperature than were the reactions involved in the primary response. In the more slowly reacting coral allografts, however, both

TABLE II

Reaction time for replicate interclone allografts of the sponge Callyspongia diffusa.

	Mean Reaction Time (days) n = 2					
	1st Set			2nd Set[a]		
Clone Combination	23°C	25°C	27°C	23°C	25°C	27°C
C1–C13	9.0	8.0	4.5	4.0	3.5	3.5
C2–C14	14.0	10.0	8.0	4.0	6.0	5.0
C3–C15	15.5	13.5	8.0	2.5	6.0	4.0
C4–C16	8.0	5.5	5.0	3.0	N.A.[c]	4.0
C5–C17	13.0	8.5	8.0	4.0	2.5	4.5
C6–C18	10.0	11.0	10.0	3.5	5.0	5.0
C7–C19	15.0	11.0	11.0	4.0	2.5	4.5
C8–C20	13.5	11.0	8.5	2.0	3.0	4.0
Median Reaction Time (days) for the 8 clone combinations	13.0	10.0	8.0	3.5	3.5	4.0
Estimate of parametric median from log–normal transformation of data[b] (days)	11.5	8.9	6.7	3.7	3.6	3.8

[a] Regrafting was accomplished after 11 days for the 27°C experiment and after 16 days in both the 25°C and 23°C experiments.

[b] From the method of Litchfield (1949).

[c] N.A.: not available since sponge C4 was completely killed by the first-set alloreaction in both replicates.

first- and second-set rejection times were temperature dependent. Extrapolating the curvilinear relationship of *M. verrucosa* first-set reaction times indicates that at temperatures below 21°C, cytotoxic allograft rejection might not occur in this coral population.

Our method of scoring definitive graft rejection obscures the fact that allogeneic killing may occur in one or both partners. The sponge combination C4–C16, for example, produced conspicuous unilateral reactions in all grafts; C4 was always killed back by over 1 mm before any sign of cytotoxic damage appeared on C16, if any appeared at all. Bilateral killing was much more common, however, indicating reciprocal interactions at the graft interfaces.

DISCUSSION

Allograft reactivity is the expression of mechanisms that normally (a) preserve the genetic integrity of the adult individual, *e.g.* preventing chimera formation that would otherwise happen if allogeneic tissues fused compatibly, and (b) promote the success of an individual genotype, sometimes at the expense of allogeneic neighbors, *e.g.* in intraspecific competition for space. Over the temperature range investigated in these experiments, the rate of this type of intraspecific immune interaction, in both coral and sponge, is temperature dependent; at higher temperatures primary transplantation rejection occurs faster. However, note the unexpectedly prolonged reaction times at 25°C, compared to 23°C, in the M1–M13, M4–M16 and M5–M17 combinations of *M. verrucosa.* Because different genotypes react quite differently to the same temperature changes, a qualitative aspect of intraspecific com-

Table III

Reaction time for replicate interclone allografts of the coral Montipora verrucosa.

	Mean Reaction Time (days) n = 2					
	1st Set			2nd Set[a]		
Clone Combination	23°C	25°C	27°C	23°C	25°C	27°C
M1–M13	16.0	19.0	13.0	15.0	9.0	4.0
M2–M14	43.0	18.0	12.0	23.0	17.0	24.0
M3–M15	21.0	12.0	10.0	16.0	9.0	4.0
M4–M16	11.0	15.0	9.0	5.0	4.0	3.0
M5–M17	17.0	18.5	10.0	9.0	9.0	6.0
M6–M18	31.0	8.0	8.0	6.0	3.0	4.0
M7–M19	29.0	12.0	14.5	11.5	12.0	4.0
M8–M20	51.5	28.0	24.0	15.0	17.0	24.0
Median Reaction Time (days) for the 8 clone combinations	21.0	15.0	10.0	12.0	9.0	4.0
Estimate of parametric median from log-normal transformation of data (days)	23.3	15.0	10.6	10.5	7.0	4.3

[a] Regrafting in the 27°C experiment was completed 16–29 days after first set grafting. The corals in the 25°C experiment were regrafted 22–29 days after the time of the first set grafting while the 23°C regraftings were made 34–55 days after the first set.

petition or adaptive immunity is also temperature dependent. Therefore, by giving a competitive or survival advantage to certain individuals, ambient sea water temperature may well affect the physical and genetic structures of coral and sponge populations.

We believe that at a given temperature the degree of allo-incompatibility, as measured by the time it takes to achieve an arbitrarily defined reaction end point, depends on the polymorphism of certain crucial gene loci. In equivalent well-studied systems in mammals, histoincompatibility depends not only on the number of mis-

Table IV

Results of paired-comparison t *tests for first and second-set mean reaction times for replicate allografts of* Callyspongia diffusa *and* Montipora verrucosa.

	Level of Significance (*P* value)	
Comparison	*C. diffusa*	*M. verrucosa*
23°C 1st set to 25°C 1st set	0.01*	0.04*
25°C 1st set to 27°C 1st set	0.02*	0.02*
23°C 2nd set to 25°C 2nd set	0.50	0.07
25°C 2nd set to 27°C 2nd set	>0.50	0.67
23°C 1st set to 23°C 2nd set	0.01*	0.01*
25°C 1st set to 25°C 2nd set	0.01*	0.01*
27°C 1st set to 27°C 2nd set	0.01*	0.06

* Significant at 95% confidence level.

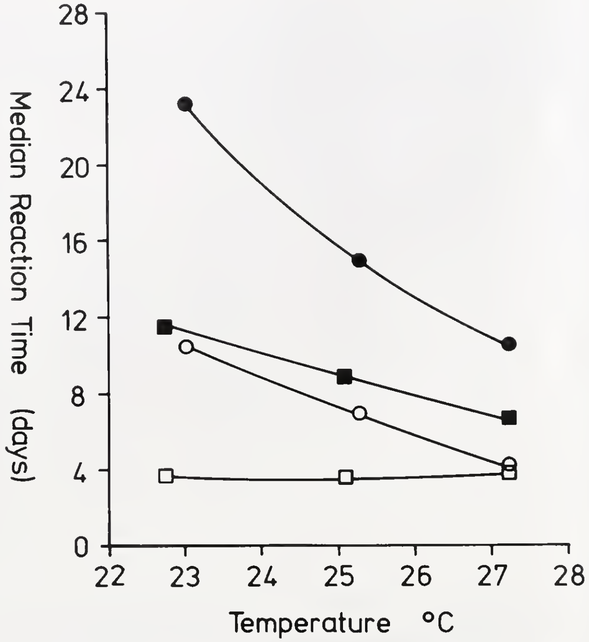


FIGURE 3. Relationship between mean morning temperature and estimated parametric median reaction time for *Montipora verrucosa* first-set grafts (solid circles), *M. verrucosa* second-set grafts (open circles), *Callyspongia diffusa* first-set grafts (solid squares) and *C. diffusa* second-set grafts (open squares).

matches at these loci but also on the individual properties of each mismatched gene; some genes, or rather their gene products, may provoke a more vigorous cytotoxic reaction either by an allogeneic graft partner, or directed at an allogeneic graft partner (Snell *et al.*, 1976). Our previous experiments with corals and sponges (Hildemann, Bigger, Johnston, *et al.*, 1980; Hildemann, Bigger, Jokiel, *et al.*, 1980; Hildemann, Johnston, *et al.*, 1980) imply that both are highly polymorphic at multiple histocompatibility loci, and also that they can sometimes be characterized either as "killers" or as particularly susceptible individuals.

Although certain genotypes may have an intraspecific competitive advantage at a given temperature, environmental temperatures fluctuate in the short and long terms. It is assumed that a high degree of genetic polymorphism within a population insures the preservation of the species despite extreme temperature perturbations (*cf.* Weins, 1977; Shick and Lamb, 1977).

Various attempts have been made to explain how diversity is maintained in coral reef environments (*e.g.*, Jackson and Buss, 1975; Buss, 1976; Connell, 1976). Although most of the discussion has been concerned with interspecific diversity, many of the concepts are transferable to intraspecific diversity (genetic polymorphism). In the absence of some intervening factors, a simple ranked hierarchy of allograft reactivity, such that individual A out-competes individual B, and B out-competes C (A→B→C), would obviously lead to eventual elimination of the lower ranked individuals. Jackson and Buss (1975) propose the possiblity of "competitive networks" in terms of different forms of competition (*i.e.* toxicity and overgrowth) such that A→B→C→A. In his work with corals on the Great Barrier Reef, Connell (1976) did not find the circular network patterns essential to the competitive network theory. His data best fit a "transitive network, external disturbances" model. In that system, A→B→C, but the higher ranked species is more susceptible to outside disturbances (*e.g.*, hurricanes). Therefore, at intervals, the number of higher-ranked individuals would decrease, allowing the lower ranked to increase, and thereby stabilize the system. However, another mechanism for maintaining diversity is suggested by earlier data and by this present study. Connell (1976; interspecific, corals), Buss (1976; interspecific, sponges, corals, bryozoans, coralline algae, foraminifera), and Bigger (1980; intraspecific, sea anemones), while showing the predictable outcome of some interactions, have noted a number of variable outcomes, i.e. A⇆B. In Connell's (1976) study, a predictable A→B only occurred in a minority of cases. The present study demonstrated a genotypically variable histoincompatible response at a given temperature and shows that such responses vary as the temperature changes. Since the experimental temperatures were well within the normal annual fluctuations of this area, annual temperature cycles could influence and shift incompatibility rankings such that A→B, A$\underset{\longrightarrow}{\leftarrow}B, A\overset{\longleftarrow}{\rightarrow}$B, A←B, or some variation, would occur sequentially. This could explain Connell's (1976) "standoffs" (inter- and intraspecific). Longer-term temperature cycles also might account for his reports of coral A overgrowing B one year, then B over A the second year, A over B the third year, etc. This kind of mechanism, which shifts competitive rankings as a consequence of normal cyclic ecological factors, could function in conjunction with major disturbances or, in some systems, could possibly maintain diversity without major external disturbances. These hypotheses of competition/ecological factor interactions require definitive field studies to substantiate their relevance to natural populations.

As a result of its niche on shallow reef tops, *C. diffusa* experiences greater temperature extremes than *M. verrucosa*. However, the more eurythermal sponge is only slighlty less sensitive than the more stenothermal coral with respect to the overall effect of temperature on primary alloreactions, i.e. both animals approximately doubled their first-set median reaction times between 27°C and 23°C. By comparison, coral growth can change 10% per 1°C (Jokiel and Coles, 1977), whereas planulation in the coral *Pocillopora damicornis* diminishes by up to an order to magnitude in response to a 1°C change from a 26–27°C optimum (Jokiel and Guinther, 1978). From field studies with three Caribbean sponges, Reiswig (1971) recorded reductions of 13–35% in overall pumping rates with an approximately 2°C decrease in ambient temperature.

Insofar as alloreactivity may be just one apsect of immunocompetence in these animals, then temperature sensitivity may also characterize other immune-type reactions. The ability to combat pathogenic organisms and the recognition of and control over populations of potential symbionts and commensals may well be reduced as temperature decreases. Low temperature immunosuppression for *M. verrucosa* from Kaneohe Bay, at about 21°C, would be significant for this animal since the low-temperature lethal limit is about 18°C (Jokiel and Coles, 1977). Therefore, temperature sensitivity of immune-type reactions could be one factor determining the restricted distribution of tropical sponges and corals.

We believe that allograft reactions are the end products of at least two basic underlying processes: allorecognition and induced cytotoxicity. Temperature may affect these processes in quite different ways, and therefore overall reaction rates may reflect complex multivariate relationships. In sponges, allograft reactions typically proceed through an intitial stage of tissue fusion (Paris, 1961; Evans *et al.*, 1980). In *C. diffusa*, we have termed this stage "tissue bridging" (Hildemann, Bigger, Johnston *et al.*, 1980) to distinguish it from long-term compatible tissue fusion; after only 4–6 hours in contact, allograft fusion is morphologically distinct from the fusion of compatible isografts. This stage probably accounts for reports of compatible allograft fusion (*i.e.* a failure of allorecognition: Moscona, 1968; McClay, 1974) especially since it may persist longer at lower temperature. Tissue bridging in *C. diffusa* rarely lasts more than 5 days at 25°C, whereas in *Hymeniacidon perleve* at 5–15°C it may persist for more than 14 days, although it is abolished at 2°C (Evans *et al.*, 1980).

The prominence of induced cytotoxicity in sponge allografts seems especially dependent on ambient temperature. Paris (1961) did not see cytotoxic reactions in orthotopic allografts of two species of Mediterranean sponge when seasonal water temperatures were lowest (11°C). However, at other times of the year, when ambient temperatures were above 16°C, the grafts and their hosts rapidly became necrotic. Van de Vyver (1980) also notes that for parabiotic allografts of *Axinella polyploides*, cytotoxic reactions are rapidly and clearly apparent to the naked eye at 23°C, but at 20°C cytotoxicity is only detectable by histological analysis, giving an initial false impression that the grafts are compatibly fused. In the present experiments, coral and sponge replicate graft pairs developed conspicuously larger areas of allogeneic killing in the 27°C treatment than in the 23°C treatment. *In situ* allograft reactions are generally more prominent in the tropics than in temperate areas.

In the future, temperature manipulation could be used to "dissect" immunological function in these animals much the same as in ectothermic vertebrates (e.g. Wright *et al.*, 1978). The different temperature sensitivity of first-set versus second-set alloreactions implies different sequences of events. The species differences in second-set reaction kinetics between corals and sponges could also imply different underlying mechanisms and controls.

ACKNOWLEDGMENT

This research was supported by NIH Grants CA 23025 and AI 15705.

LITERATURE CITED

BIGGER, C. H. 1980. Interspecific and intraspecific acrorhagial aggressive behavior among sea anemones, a recognition of self and not-self. *Biol. Bull.* **159:** 117–134.

BUSS, L. W. 1976. Better living through chemistry: the relationship between allelochemical interactions and competitive networks. Pp. 315–327 in F. W. Harrison and R. R. Cowden, Eds., *Aspects of sponge biology.* Academic Press, New York.

CLAUSEN, C. D., AND A. A. ROTH. 1975. Effect of temperature and temperature adaptation on calcification rate in the hermatypic coral *Pocillopora damicornis. Mar. Biol.* **33:** 93–100.

COLES, S. L., AND P. L. JOKIEL. 1977. Effects of temperature on photosynthesis and respiration in hermatypic corals. *Mar. Biol.* **43:** 209–216.

COLES, S. L., AND P. L. JOKIEL. 1978. Synergistic effects of temperature, salinity and light on the hematypic coral *Montipora verrucosa. Mar. Biol.* **49:** 187–195.

CONNELL, J. H. 1976. Competitive interactions and the species diversity of corals. Pp. 51–58 in G. O. Mackie, Ed., *Coelenterate ecology and behavior.* Plenum Press, New York.

CURTIS, A. S. G. 1979. Individuality and graft rejection in sponges, a cellular basis for individuality in sponges. Pp. 39–48 in G. Larwood and B. R. Rosen, Eds., *Biology and systematics of colonial organisms.* Academic Press, New York.

EVANS, C. W., J. KERR, AND A. S. G. CURTIS. 1980. Graft rejection and immune memory in marine sponges. Pp. 27–34 in M. J. Manning, Ed., *Phylogeny of immunological memory.* Elsevier/North Holland Biomedical Press, Amsterdam.

HILDEMANN, W. H. 1962. Immunogenetic studies of poikilothermic animals. *Am. Nat.* **96:** 195–204.

HILDEMANN, W. H., C. H. BIGGER, I. S. JOHNSTON, AND P. L. JOKIEL. 1980. Characteristics of transplantation immunity in the sponge, *Callyspongia diffusa. Transplantation,* **30:** 362–367.

Hildemann, W. H., C. H. Bigger, P. L. Jokiel, and I. S. Johnston. 1980. Characteristics of immune memory in invertebrates. Pp. 9–15 in M. J. Manning, Ed., *Phylogeny of immunological memory*. Elsevier/North Holland Biomedical Press, Amsterdam.

Hildemann, W. H., I. S. Johnston, and P. L. Jokiel. 1979. Immunocompetence in the lowest metazoan phylum: transplantation immunity in sponges. *Science* **204:** 420–422.

Hildemann, W. H., P. L. Jokiel, C. H. Bigger, and I. S. Johnston. 1980. Allogeneic polymorphism and alloimmune memory in the coral, *Montipora verrucosa. Transplantation,* **30:** 297–301.

Hildemann, W. H., R. L. Raison, G. Cheung, C. J. Hull, L. Akaka, and J. Okamoto. 1977. Immunological specificity and memory in a scleractinian coral. *Nature* **270:** 219–223.

Hildemann, W. H., R. L. Raison, and C. J. Hull. 1977. Immunocompetence in corals: issues of specificity, memory and mechanisms. Pp. 9–16 in J. B. Solomon and J. D. Horton, Eds., *Developmental immunobiology*. Elsevier/North Holland Biomedical Press, Amsterdam.

Houck, J. E., R. W. Buddemier, S. V. Smith, and P. L. Jokiel. 1977. The response of coral growth rate and skeletal strontium content to light intensity and water temperature. Pp. 425–431 in D. L. Taylor, Ed., *Proc. Third Int. Symp. Coral Reefs*, Vol. 2. Rosenstiel School of Marine and Atmospheric Sciences, Coral Gables, Florida.

Jackson, J. B. C. and L. Buss. 1975. Allelopathy and spatial competition among coral reef invertebrates. *Proc. Nat. Acad. Sci. U. S. A.* **72:** 5160–5163.

Johnston, I. S., and W. H. Hildemann. In press. Cellular defense systems in the Porifera. In N. Cohen and M. Sigel, Eds., *The ontogeny and phylogeny of the reticuloendothelial system.* Plenum Press, New York.

Jokiel, P. L., and S. L. Coles. 1977. Effect of temperature on the mortality and growth of Hawaiian reef corals. *Mar. Biol.* **43:** 201–208.

Jokiel, P. L., and E. B. Guinther. 1978. Effects of temperature on reproduction in the hermatypic coral *Pocillopora damicornis. Bull. Mar. Sci.* **28:** 786–789.

Lang, J. 1973. Interspecific aggression by scleractinian corals. 2. Why the race is not only to the swift. *Bull. Mar. Sci.* **23:** 260–279.

Litchfield, J. T. 1949. A method for rapid graphic solution of time-per cent effect curves. *J. Pharmacol. Exp. Ther.* **97:** 399–408.

Manning, M. J., and R. J. Turner. 1976. *Comparative immunobiology*. Halstead Press/John Wiley & Sons, New York. 184 pp.

McClay, D. R. 1974. Cell aggregation: properties of cell surface factors from five species of sponge. *J. Exp. Zool.* **188:** 89–102.

Moscona, A. A. 1968. Cell aggregation: properties of specific cell ligands and their role in the formation of multicellular systems. *Dev. Biol.* **18:** 250–277.

Paris, J. 1961. Greffes et sérologie chez les éponges silicieuses. *Vie Milieu Suppl.* **11:** 1–74.

Raison, R. L., C. J. Hull, and W. H. Hildemann. 1976. Allogeneic graft rejection in *Montipora verrucosa*, a reef-building coral. Pp. 3–8 in R. K. Wright and E. L. Cooper, Eds., *Phylogeny of thymus and bone marrow—bursa cells.* Elsevier/North Holland Biomedical Press, Amsterdam.

Reiswig, H. M. 1971. *In situ* pumping activities of tropical Demospongiae. *Mar. Biol.* **9:** 38–50.

Sarà, M. and J. Vacelet. 1973. Ecologie des démosponges. Pp. 462–576 in P. P. Grassé, Ed., *Traité de zoologie, anatomie, systematique, biologie: Spongiaires.* Masson, Paris.

Shick, J. M., and A. N. Lamb. 1977. Asexual reproduction and genetic population structure in the colonizing sea anemone *Haliplanella luciae. Biol. Bull.* **153:** 604–617.

Snell, G. D., J. Dausset, and S. Nathenson. 1976. *Histocompatibility*. Academic Press, New York. 401 pp.

Trench, R. K. 1979. The cell biology of plant-animal symbiosis. *Ann. Rev. Plant Physiol.* **30:** 485–531.

Van de Vyver, G. 1980. Second-set allograft rejection in two sponge species and the problem of alloimmune memory. Pp. 15–26 in M. J. Manning, Ed., *Phylogeny of immunological memory.* Elsevier/North Holland Biomedical Press, Amsterdam.

Vaughan, T. W. 1907. Recent Madreporaria of the Hawaiian Islands and Laysan. *U. S. Nat. Mus. Bull.* **59:** 1–427.

Weins, J. A. 1977. On competition and variable environments. *Am. Scientist* **65:** 590–604.

Wright, R. K., E. F. Eipert, and E. L. Cooper. 1978. Regulating role of temperature on the development of ectothermic vertebrate lymphocyte populations. Pp. 80–92 in M. E. Gershwin and E. L. Cooper, Eds., *Animal models of comparative and developmental aspects of immunity and disease.* Pergamon Press, New York.

Reference: *Biol. Bull.*, **160**: 292–302. (April, 1981)

PHENOTYPIC VARIATION IN THE CORAL *MONTASTREA CAVERNOSA* AND ITS EFFECTS ON COLONY ENERGETICS

HOWARD R. LASKER*

Department of the Geophysical Sciences, University of Chicago, Chicago, Illinois 60637

ABSTRACT

Variation in polyp expansion; zooxanthellae density; coloration; and polyp shape, size, and density can be used to divide shallow water (<20 m) colonies of *Montastrea cavernosa* into two morphs, diurnal and nocturnal. Respiration rates of the morphs are related to the number and size of polyps, an index of biomass. Small changes in the size or number of polyps, while not affecting the size of the whole colony, affect respiration rates. The diurnal morph has greater zooxanthellae densities than the nocturnal morph and correspondingly greater rates of gross primary production. Respiration and gross primary production are both increased by expansion of the polyps. Colonies with low rates of gross primary production are characterized by morphologies and behaviors that reduce maintenance costs. This suggests that limitations of primary production play a major role in the development (and/or evolution) of *M. cavernosa*.

INTRODUCTION

The role of zooxanthellae in reef corals has been debated extensively over the last 75 years. It has become increasingly clear that photosynthate from the zooxanthellae provide much or most of the energetic needs of many (if not all) reef corals (see Muscatine, 1973; Muscatine and Porter, 1977; McCloskey *et al.*, 1978). However, the role of zooxanthellae in shaping the behavior and morphology of reef corals remains unclear.

This study examines the effects of morphology and behavior upon the primary production of *Montastrea cavernosa* (Linnaeus). From such an analysis it is possible to determine the extent to which morphology and behavior in *M. cavernosa* colonies maximizes primary production. The study provides an understanding of the factors controlling productivity and also provides a first approximation of the selective importance of primary production in determining colony phenotype.

M. cavernosa is particularly suited for such an analysis. It is common in the fauna of most Caribbean reefs and exhibits a striking variety of phenotypes both within and between habitats. Thus the effects of phenotypic variation can be examined using colonies from both similar and dissimilar environments.

In Panama, differences among *M. cavernosa* colonies have led to the distinction of two morphs, referred to as the large and small polyp forms (Lehman and Porter, 1973) or the diurnal and nocturnal morphs (Lasker, 1976, 1977, 1979). The present study uses the diurnal-nocturnal classification of Lasker (1979), which distinguishes those colonies whose polyps are most commonly expanded during the day, the diurnal morph, from those colonies whose polyps are never expanded during the day, the nocturnal morph. The polyps of both morphs are fully expanded at night.

Received 17 September 1980, accepted 21 January 1981.

* Current Address: Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260.

METHODS

Laboratory experiments were conducted at the Galeta Marine Laboratory of the Smithsonian Tropical Research Institute in Panama. Field observations and collections of *M. cavernosa* were made in four areas along Panama's Caribbean coast: at Galeta Island (east of the Panama Canal), Buena Ventura Island (at the mouth of Portobelo Harbor), the San Blas Islands, and the Bocas del Toro region.

The morphologic and behavioral data presented here are based on observations of colonies encountered on "distribution dives" at each study site. During these dives all of the *M. cavernosa* colonies found within 3 m of the 2, 5, 10, and 15 m depth contours were examined and data collected concerning the colonies' expansion, polyp size, polyp height, colony shape, and color. At six sites closeup photographs were taken of every second coral. Equal areas were sampled at each depth, but area sampled differed between sites due to differences in the size of the reefs. At some localities, where the reef extended below 15 m, deeper depths were sampled.

Polyp size, in most cases, was scored as large or small depending on whether calice diameter was less than or greater than 6 mm. Polyp size was usually apparent at first observation, since most colonies with large polyps had mean diameters above the 6 mm cutoff (Fig. 1). In cases of uncertainty, 5–10 polyps were measured with a ruler and an average determined. If polyp size was still uncertain ($\bar{x} \simeq 6$ mm) the colony was classified indeterminent and excluded from the analysis. Only 7% of the 671 colonies examined were classified indeterminent.

Accuracy of the field observations was tested by comparing diameters of the 218 photographed colonies to their field classifications. Mean size was calculated from the diameters of 20 polyps measured (±0.1 mm) from the projected negative of each photograph. Comparison of the two types of polyp size data indicates that 80% of the measured colonies were correctly classified. Closeup photographs were also analyzed for polyp density, by determining the number of polyps per 20 cm^2 of colony surface area.

Polyp height is the vertical distance from the coenosteum to the lip of the calice. Among *M. cavernosa* colonies three distinct states were observed: short, intermediate, and tall. Colonies with short polyps had almost flat surfaces (measured polyp height $\simeq$ 1mm) without distinct polyp walls. Colonies with intermediate polyps had irregular surfaces (polyp height, 2–4 mm) and displayed distinct polyp walls. Col-

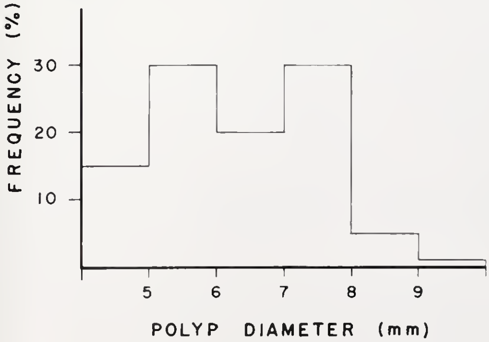


FIGURE. 1. Size frequency distribution of mean polyp diameter. The distribution is based on measurements of 218 colonies from Portobelo and the San Blas Islands. Nocturnal morph colonies usually had mean polyp diameters > 6 mm and diurnal morph colonies < 6 mm.

onies with tall polyps had corallites which distinctly stood out from the coenosteum (polyp height 3–7 mm).

Coenosarc color was scored by noting five apparently distinct colors: red, blue, green, brown, and a white ectodermal coloration. The brown color, which varied in intensity, was recorded as being absent, present, or abundant. The oral disc was usually the same color as the coenoosarc, but in some cases contained other colors. The presence or absence of both a white ectodermal coloring and a "fluorescent" green coloring on the oral disc was also noted.

Weights of polyp and coenosarc tissue were determined from preserved specimens decalcified in 5% HCl. Plugs of tissue then were removed with a cork borer, blotted dry, and weighed on an analytic balance.

Zooxanthellae densities were also determined from preserved specimens. Plugs of tissue from polyp and coenosarc were removed with a cork borer and homogenized in a tissue grinder. The number of zooxanthellae per milliliter of homengenate was then determined from replicate counts made with a hemocytometer. (See Lasker, 1977, for further details.)

Photosynthesis and respiration rates were determined by observing oxygen fluxes of 18 colonies in a closed chamber respirometry unit in light and darkness. Colonies were collected from virtually identical micro-habitats at 8–10 m depth on a fringing reef north of Buena Ventura Island. Colonies collected were small enough to fit in the 10 cm diameter respirometric chamber and were largely, but not wholly, free of encrusting and boring organisms. No adjustments were made for the respiratory activity of these forms.

Colonies were transported from the field to the laboratory in a darkened cooler and were always kept in sea water. The colonies were allowed to acclimate in shaded sea tables for 2–3 days and were checked for normal behavior and appearance before respirometric measurement. Colonies were classified nocturnal or diurnal on the basis of behavior at the time of collection and in the laboratory.

Respirometric measurements began within 5 days of collection. During the 5 to 6 days of measurement the colonies were kept in a constant temperature water bath at 28°C and were exposed to 12 h of 25 μEinsteins$\cdot m^{-2} \cdot s^{-1}$ illumination each day. Measurements were made as follows: Pairs of colonies, usually one of each morph, were measured in tandem. The colonies were placed in glass chambers between 0800 and 0900 h and were allowed to acclimate in the dark for several hours. During the acclimation, sea water was pumped into the chamber from the surrounding sea water bath, providing circulation within the chamber and preventing the accumulation of metabolites.

In order to measure oxygen flux, exchange with the sea water bath was discontinued; sea water from the chamber was pumped past a Hydrolab TDO-2 polarographic oxygen sensor and then returned to the chamber. Dissolved oxygen content within the chamber was monitored in this manner for 35 min. For analysis the 35 min sampling intervals were divided into one 5-min initialization and two 15-min measurement periods. After the first colony was measured the pumping system was rearranged to measure oxygen flux of the second colony. Flow through the chamber during measurements was maintained at 3 l/min, which resulted in a florescein dye residence time of about 90 s. The water in the chamber not being measured was exchanged with the sea water bath.

At the end of each day's measurements the colonies were removed and a measurement made of oxygen flux of the empty chamber. This value was then used to correct all of that day's measurements. The 190 l of sea water in the water bath was replaced daily, and after several days of operation the entire system was drained, washed with 50% ethanol, and dried.

Photosynthesis of contracted colonies was measured at light intensities of 25, 93, and 1890 μEinsteins $\cdot m^{-2} \cdot s^{-1}$. Polyp expansion of the colony was noted at the start and finish of each 15 min period. In cases where polyp closure was desired the chamber was rocked until the polyps contracted. Colonies did not always expand fully during the experiment. Consequently, some were measured repeatedly to obtain large enough sample sizes. Two morning and two afternoon measurements (35 min each) were made at each light intensity. Dark-chamber measurements alternated with light measurements, and additional dark-chamber measurements were made at night. Measurements at 1890 μEinsteins $\cdot m^{-2} \cdot s^{-1}$ were always made the last day of the experiments in order to avoid non-reversible photoinhibition. Light at the two lower intensities was provided with cool white fluorescent bulbs, supplemented with incandescent flood lights for the high light intensity. Light measurements were made with a Lambda Instruments quantum flux sensor (LI-190).

Following the measurements, colonies were preserved in formalin. Surface area was measured by carefully wrapping aluminum foil over the colony and then determining the area of aluminum foil used from its weight. Volume of the corallum was determined by displacement in water, and then subtracted from the measurement chamber volume to determine the amount of sea water present during the experiment and thereby to calculate oxygen flux. The diameters of the calices of 20 polyps were measured to the nearest 0.05 mm with a vernier caliper and the means (reported here as polyp size) calculated. The number of polyps on each colony was also counted.

RESULTS

Morphologic variation

M. cavernosa's morphs were defined on the basis of their expanison behavior, but they are also distinguishable on the basis of a number of morphological characters, most conspicuously polyp size. Mean polyp diameters of randomly selected colonies were bimodally distributed (Fig. 1) and large polyps were most commonly associated with nocturnal morph colonies (Table 1). Nocturnal morph colonies also had lower polyp densities (0.5–1.0 polyps/cm^2) and more frequently had tall polyps than the diurnal morph (Table I). The diurnal morph had small polyps, higher polyp densities (1.5–2.0 polyps/cm^2), and intermediate polyp height. Diurnal morph colonies were more commonly planar in shape, while nocturnal morph colonies were more commonly nodular. The diurnal morph also tended to have brown coloration, and approximately 50% of diurnal morph colonies had brilliant green oral discs.

Relationships between morphology and behavior were most pronounced at depths less than 15 m. At greater depths daytime expansion became less common (Fig. 2). Increasing depth also was related to a decrease in polyp density (polyps/cm^2) independent of polyp diameter (Fig. 3).

Polyps had greater zooxanthellae densities than coenosarc among both morphs (diurnal morph—6.65 [±0.36] vs. 3.39 [±0.16] 10^6 cells/cm^2 [±standard error]; nocturnal morph—4.72 [±0.56] vs. 2.93 [±0.17] 10^6 cells/cm^2). Polyps of the diurnal morph also had greater zooxanthellae densities than those of the nocturnal morph ($p < 0.05$, Mann-Whitney U test).

TABLE I

Contingency tables showing the relationship between daytime expansion and morphologic traits among colonies from reefs along Panama's Caribbean coast. See text for explanation of polyp size and polyp height.

		Daytime Polyp Expansion		Significance of χ^2-test
		contracted	expanded	
Polyp size	large	204	30	$P \ll 0.001$
	small	125	182	
Polyp height	tall	43	15	$P \ll 0.001$
	intermediate	117	173	
	short	207	37	
Colony shape	planar	122	131	$P \ll 0.001$
	nodular	229	85	
Brown coenosarc pigment	abundant	162	154	$P \ll 0.001$
	present	85	37	
	absent	111	29	
Green coenosarc pigment	present	133	49	$P < 0.005$
	absent	224	170	
White oral disc	present	23	36	$P < 0.002$
	absent	275	183	
Green oral disc	present	16	110	$P \ll 0.001$
	absent	283	106	

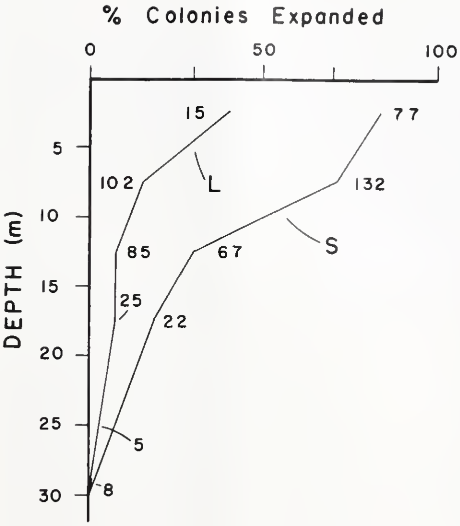
Respirometric measurements

Mean rates of respiration and gross primary production of each of the 18 colonies are presented in Table II. Variability among respiration rates of the colonies was great and no statistically significant differences between morphs were found. Respiration rates of colonies were significantly greater when polyps were expanded ($p = 0.01$, randomization test; Siegel, 1956). Unlike respiration rates, gross primary productivity of the diurnal morph was significantly greater than that of the nocturnal morph at all three light intensities ($p < 0.05$, Mann-Whitney U test). Gross primary production of diurnal morph colonies at 25 μEinsteins$\cdot m^{-2} \cdot s^{-1}$ was significantly greater when their polyps were expanded ($p = 0.027$, randomization test). The nocturnal morph could not be induced to expand in the light and consequently the effects of expansion on it could not be ascertained.

DISCUSSION

Effect of morphology and behavior on primary production

Colonies' and morphs' differing rates of respiration and photosynthesis (Table II) do not reflect different physiologies; rather, they are consequences of the colonies' differing morphologies. The effects of morphology are best illustrated by respiration rates. Respiration by any organism can be expected to vary with the organisms' biomass. Respiration rates presented in Table II are adjusted for the colonies' differing surface areas, an index of biomass. However, surface area alone is a poor estimator of colony biomass. Polyps were more massive than coenosarc (Fig. 4). Therefore, an accurate characterization of colony biomass must take the size and number of polyps into account. In Figure 5 respiration is plotted as a function of polyp area (the percent of the colony surface occupied by polyps). Polyp



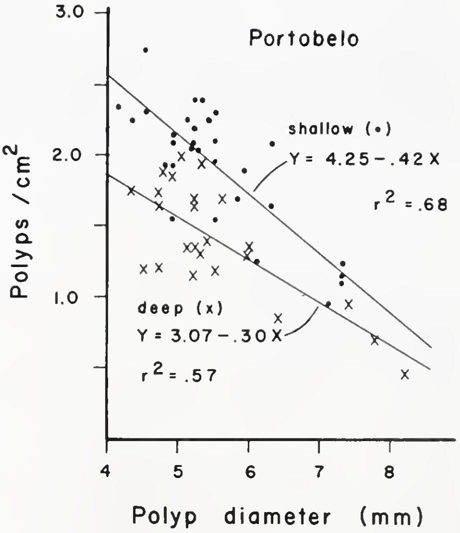


FIGURE 2. (Left) Proportion of colonies with large polyps (L) and with small polyps (S) found expanded in each of five different depth zones. Values based on transects from the San Blas Islands and Bocas del Toro regions of Panama. Values in parenthesis indicate numbers of colonies used to derive each data point, and roughly correspond to the colonies' relative abundances at the different depths.

FIGURE 3. (Right) Least squares regressions of polyp size on polyp density at 8 and 20 m depth from Portobelo. Mean diameter of polyps from the two populations do not differ, but polyp density of the deep population (20 m) is significantly lower than that of the shallow population (8 m) ($p < 0.05$, ANOVA).

area is an index of biomass per unit area and accounts for much of the variance in the respiration rates.

The importance of polyp size and number was again evident when data on whole colony respiration rates were analyzed. Linear regressions were determined between respiration and two measures of colony size:

$$R_T = 16.36A + 51.82, \qquad r^2 = 0.64 \qquad (1)$$

$$R_T = 52.79A_p + 51.82, \qquad r^2 = 0.74, \qquad (2)$$

where R_T is total respiration (μg O_2/h), A is total colony surface area (cm^2), and A_p is area of the polyps (cm^2). A greater portion of variance in respiration rates is explained by (2), which uses the area of the polyps as an estimator of biomass.

The solution of (2) was calculated in a stepwise linear regression procedure which considered, but rejected as insignificant, the effects of coenosarc area. This does not necessarily mean coensarc makes no contribution to respiration rate. Coenosarc and polyp areas among the 18 colonies were correlated ($r = 0.64$, $p < 0.01$), and the covariance between the areas of the two makes it difficult to discuss whether the two actually make an independent contribution to respiration. As a consequence of the correlation between polyp and coenosarc areas it is likely that the effect of coenosarc in (2) is already incorporated into the term for polyp area.

The contribution of coenosarc to the respiration rate can be tested in data sets

Table II

Results of the respirometry experiments. Mean values of gross primary productivity and respiration are expressed in $\mu g\ O_2 \cdot cm^{-2} \cdot h^{-1}$. *Respiration values are net flux for dark chamber measurements, and gross primary production is net flux in light plus dark chamber loss. The number of replicates giving each mean is in parenthesis. Dashes (—) indicate no measurements were taken.*

	Respiration		Gross Primary Productivity (Light Intensity (μEinsteins·m^{-2}·s^{-1}))			
			(25)		(93)	(1890)
	expanded	contracted	expanded	contracted	contracted	contracted
Diurnal Morph						
PBX-1	22.01 (5)	13.45 (18)	25.68 (4)	23.23 (4)	23.23 (6)	41.57 (4)
PBX-3	17.44 (8)	10.90 (16)	26.16 (6)	15.26 (4)	28.33 (6)	46.87 (4)
PBX-5	17.10 (10)	11.40 (16)	15.20 (4)	13.30 (8)	30.40 (4)	45.60 (2)
PBX-7	—	14.81 (16)	—	14.43 (4)	18.23 (4)	27.34 (2)
PBX-9	22.98 (6)	20.84 (20)	22.99 (4)	16.57 (8)	30.46 (4)	51.83 (4)
PBX-11	23.66 (2)	19.32 (20)	22.08 (2)	19.71 (6)	24.84 (4)	33.51 (4)
PBX-13	24.71 (2)	23.21 (18)	13.48 (2)	15.81 (2)	42.68 (4)	45.67 (4)
PBX-15	—	17.11 (16)	—	16.72 (4)	26.36 (4)	41.61 (4)
PBX-19	25.26 (2)	24.06 (6)	22.56 (2)	21.08 (6)	—	—
PBX-21	34.37 (4)	19.03 (4)	30.69 (5)	20.26 (3)	—	—
mean	23.10	17.41	22.36	17.64	28.06	41.75
Nocturnal morph						
PBX-2	17.63 (2)	13.4 (25)	—	12.34 (4)	24.33 (4)	22.57 (8)
PBX-4	16.59 (4)	15.72 (18)	—	15.28 (4)	19.21 (6)	27.07 (4)
PBX-6	—	9.39 (16)	—	8.54 (4)	16.50 (4)	37.84 (4)
PBX-8	11.65 (08)	12.76 (8)	—	7.21 (4)	26.63 (4)	40.50 (4)
PBX-10	29.95 (6)	21.54 (15)	—	11.02 (4)	19.54 (4)	26.5 (4)
PBX-12	—	23.21 (23)	—	2.36 (4)	21.20 (4)	29.11 (4)
PBX-14	—	18.78 (20)	—	6.4 (4)	27.32 (4)	41.83 (4)
PBX-16	—	15.59 (16)	—	15.05 (4)	18.28 (4)	26.34 (4)
mean	18.96	16.30		9.78	21.63	31.48

where polyp and coenosarc areas are not correlated. Respiration rates with expanded polyps could not be obtained for some colonies. Among this subset of colonies coenosarc and polyp areas were not correlated. The analogous solution of (2) among these colonies includes a term for coenosarc area (A_c).

$$R'_T = 44.69A_p + 18.21A_c – 285.56, \qquad r^2 = 0.78 \tag{3}$$

In the analysis presented above the best estimators of respiration are those which most accurately reflect differences in colony biomass. Among colonies of *M. cavernosa*, small morphologic differences that did not affect the size of the colony did affect biomass and respiration rates.

Like respiration, gross primary production is affected by morphology. But to explain variance in gross primary production one must also consider light level and the number of zooxanthellae in the colony.

Gross primary production data were fitted to a simple Michaelis-Menton model.

$$GPP(I) = 37.1I/(42.4 + I), \qquad r^2 = 0.64, \tag{4}$$

where GPP(I) is gross primary production ($\mu g\ O_2 \cdot cm^{-2} \cdot h^{-1}$) at light intensity

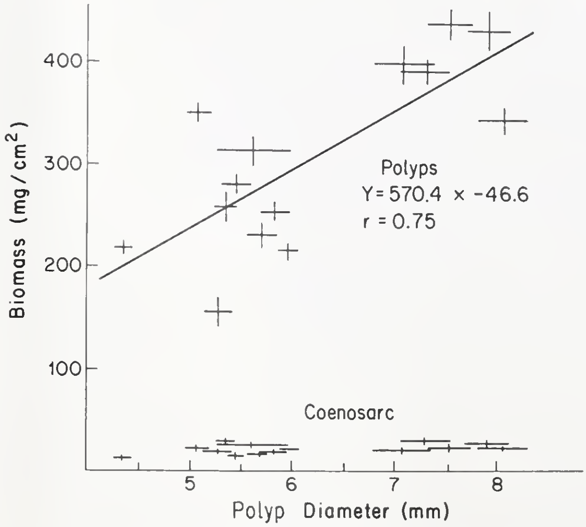


FIGURE 4. Biomass (preserved wet weight per cm^2) as a function of polyp diameter. The upper line presents the least squares linear regression of polyp diameter on polyp weight. The lower cluster of points are for coenosarc. Values are presented as means ± 1 standard error for each colony.

I (μEinsteins $\cdot m^{-2} \cdot s^{-1}$). Some of the remaining variance can be attributed to the significant differences between gross primary production of the diurnal and nocturnal morphs. Separate analysis of the two morphs improves the goodness of fit

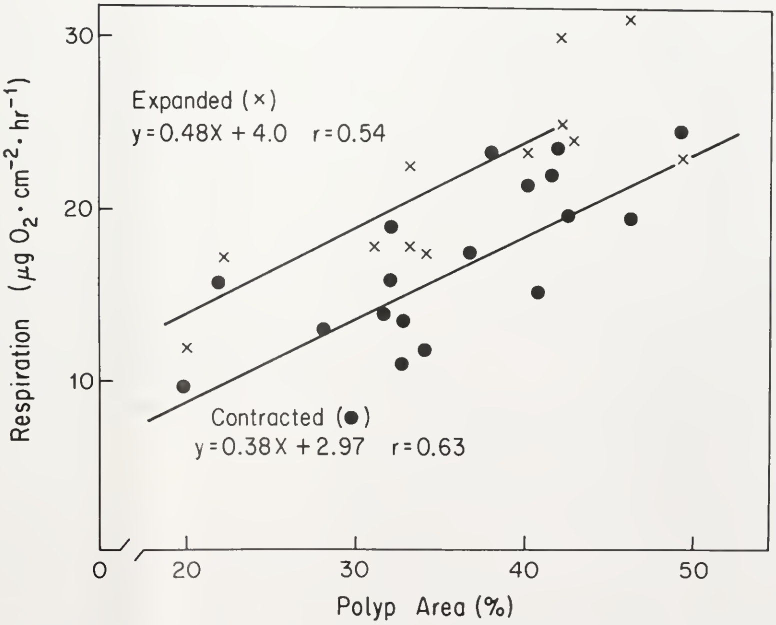


FIGURE 5. Relationship between respiration rate and polyp area of expanded (×) and contracted (dot) colonies of *M. cavernosa*.

of the Michaelis-Menton model,

$$\mathrm{GPP(I)}_{\mathrm{Diurnal}} = (42.0)\mathrm{I}/(38.4 + \mathrm{I}), \qquad r^2 = 0.73 \tag{5}$$

$$\mathrm{GPP(I)}_{\mathrm{Nocturnal}} = (32.5)\mathrm{I}/(51.2 + \mathrm{I}), \qquad r^2 = 0.74 \tag{6}$$

The differences in gross primary production of the two morphs can be attributed to differences in their zooxanthellae densities. The diurnal morph colonies had greater zooxanthellae densities within polyps and greater polyp areas. (Zooxanthellae densities were not measured in the 18 colonies used in the respirometry experiments, and the extrapolation is based on the average values derived from Lasker, 1977). Correction for zooxanthellae density improves the goodness of fit and also incorporates data from both morphs into a single model,

$$\mathrm{GPP(I)} = (\mathrm{Z})(\mathrm{I})(91.4)/(42.4 + \mathrm{I}), \qquad r^2 = 0.76, \tag{7}$$

where Z is zooxanthellae density (10^6 cells/cm^2). Although polyp area affects the total number of zooxanthellae, the variance in polyp areas among these 18 colonies only accounted for 1% of the variance in gross primary production. Unlike respiration rates, differences in polyp area among *M. cavernosa* colonies contributed little to the observed differences in gross primary production.

Equations (4)–(7) account for variation between colonies and morphs, but do not explain the increase in gross primary production with polyp expansion. Sebens and de Riemer (1978) suggested that this increase could result from a greater diffusive supply of limiting nutrients like ammonium. Lasker (1977), on the other hand, has suggested that contracted polyps shade their zooxanthellae, which reduces their photosynthetic activity. The coenosarc is comparable in zooxanthellae density and tissue thickness to the superficial layer of a contracted polyp. Absorbance of the coenosarc can, therefore, provide an estimate of shading in the polyp. Coenosarc light absorbance was estimated by placing five replicate pieces of decalcified coenosarc over a quantum flux sensor (Lamda LI-190) and observing the decrease in light reception. Mean transmissivity of coenosarc for the three colonies so tested (five replicates each) were 0.22, 0.22, and 0.12. (The low value was from a colony with white ectodermal coloration. None of the colonies used in the respirometry experiments was so pigmented.) This suggests that contracted polyps may reduce light reception by close to 80%. Crossland and Barnes (1977) have argued that because photosynthesis by zooxanthellae becomes saturated at low levels, shading should be unimportant. In this study the effects of expansion were tested at 25 μEinsteins $\cdot m^{-2} \cdot s^{-1}$. Given the light level and a half-saturation constant of 42.4 μEinsteins $\cdot m^{-2} \cdot s^{-1}$ (from [7]), an 80% reduction in light to zooxanthellae in the tentacles and oral disk should cause a 73% reduction in their productivity. Using the known zooxanthellae densities and mean polyp area of the diurnal morph colonies (39% of total surface area) a predicted reduction in the gross primary production of contracted colonies can be calculated. The predicted value is 20%; the observed reduction was 21% (Table II).

The role of primary production in determining phenotype

Spencer-Davies (1977) estimated that primary production in *M. cavernosa* accounts for 49–102% of a colony's energetic requirements. Clearly, primary production plays a significant role in *M. cavernosa's* energetic budget. However, the relationship between primary productivity and particulate feeding is not clear. Polyp size, density, and expansion affect a colony's ability to capture particulates as well as its primary production. Therefore, the presence of differing phenotypes among

M. cavernosa colonies can provide insights about the role of "autotrophy and heterotrophy," (sensu Porter, 1976).

What occurs when primary production is altered independent of morphology and behavior? Do changes occur in ontogeny (or have they occurred evolutionarily) which compensate for the change in energy balance? Or are the factors controlling phenotype in *M. cavernosa* independent of energetic considerations? These questions can be examined in two instances: where primary production changes with increasing depth, and where primary production changes with different zooxanthellae densities.

Spencer-Davies (1977) observed a decrease in *M. cavernosa* gross primary production with depth in Jamaica. This is paralleled by a decrease in respiration rate, some of which may be attributed to reduced biomass (Spencer-Davies, 1980). In Panama, one of the principal effects of depth was a reduction in polyp density (Fig. 2) and therefore in biomass. Although reduced polyp density lowers colony maintenance costs, it also should reduce particulate capture (*i.e.,* fewer capture organs).

The proportion of colonies expanded during the day also decreases with depth (Fig. 3). Like reduced polyp density, the loss of daytime expansion lowered respiration rates and can be expected to reduce particulate capture (less time spent feeding). But contraction of the polyps also reduced gross primary production. Therefore, the net energetic effect of contraction is dependent on changes to both respiration and gross primary production. At 25 μEinsteins $\cdot m^{-2} \cdot s^{-1}$ contraction effects a 24% reduction of respiration rate and a 21% reduction of gross primary production (Table II). At higher light levels, which occur through most of the day, the effect of contraction on gross primary production is reduced. Therefore, in most cases polyp contraction increases net primary production. Like decreased polyp density, daytime contraction conserves energy but should also reduce particulate feeding.

The lower zooxanthellae densities of the nocturnal morph reduces its gross primary production. As in the case of increasing depth, the nocturnal morphs' reduced primary production is correlated with daytime contraction and with low polyp density. Even after compensating for its larger polyps the nocturnal morph had lower polyp areas than the diurnal morph. As noted, these characters lower colony maintenance costs but should reduce particulate feeding. The nocturnal morph also has large polyps. Large polyps increase biomass and respiration but also increase zooplankton capture ability (Lasker, 1976).

Morphologic and behavioral variability among *M. cavernosa* colonies follows two patterns. The nocturnal morph, which has lower rates of gross primary production, has larger polyps, which will enhance zooplankton feeding. In this manner the morphs of *M. cavernosa* display complementarity between autotrophy and heterotrophy like that proposed by Porter (1976).

On the other hand, other morphologies and behaviors which also enhance particulate feeding are not complementary to primary production. Instead, they are positively correlated with it. This suggests that the benefits of daytime expansion and increased polyp density only exceed their costs at high levels of primary production. Dependence of these characters on primary production implies that their benefits are not energetic. Rather, the benefits may involve non-energetic aspects of particulate feeding (like nitrogen supply) or may involve aspects of *M. cavernosa's* ecology that are independent of feeding (sediment removal, for example; Lasker, 1980).

The level of gross primary production among *M. cavernosa* colonies correlates strongly with trends in phenotypic variation. This indicates the important role that the zooxanthellae have played in the evolution and development of *M. cavernosa*. However, the complexity of the interactions between primary production and phenotype in this species suggests the relationship between "autotrophy and heterotrophy" among reef corals cannot be understood if colony energetics alone are examined.

ACKNOWLEDGMENTS

This manuscript is based on a Ph.D. thesis, Department of the Geophysical Sciences, University of Chicago, and was further developed during a Rosenstiel Fellowship at the Rosenstiel School of Marine and Atmospheric Science, University of Miami. The research was supported by the Doherty Foundation (Smithsonian Tropical Research Institute), the Henry Hinds Fund for Evolutionary Biology (University of Chicago), and National Science Foundation Grant OCE-77-00148. The comments of E. G. Leigh, Jr., D. L. Taylor, and two anonymous reviewers have been helpful, as have been the discussions and assistance of T. J. M. Schopf, G. L. Hendler, and numerous colleagues from both the Smithsonian Tropical Research Institute and the University of Chicago. To all I am grateful.

LITERATURE CITED

CROSSLAND, C. J., AND D. J. BARNES. 1977. Gas-exchange studies with the Staghorn coral *Acropora acuminata* and its zooxanthellae. *Mar. Biol.* **40:** 185–194.

LASKER, H. R. 1976. Intraspecific variability of zooplankton feeding in the hermatypic coral *Montastrea cavernosa*. Pp. 101–109 in G. W. Mackie, Ed., *Coelenterate Ecology and Behavior*. Plenum Press, New York and London.

LASKER, H. R. 1977. Patterns of zooxanthellae distribution and polyp expansion in the reef coral *Montastrea cavernosa*. Pp. 607–613 in D. L. Taylor, Ed., *Proceedings, Third International Coral Reef Symposium*, Vol. 1. University of Miami, Miami, FL.

LASKER, H. R. 1979. Light dependent activity patterns among reef corals: *Montastrea cavernosa*. *Biol. Bull.* **156:** 196–211.

LASKER, H. R. 1980. Sediment rejection by reef corals: the roles of behavior and morphology in *Montastrea cavernosa* (Linnaeus). *J. Exp. Mar. Biol. Ecol.* **47:** 77–87.

LEHMAN, J. T., AND J. W. PORTER, 1973. Chemical activation of feeding in the Caribbean reef building coral *Montastrea cavernosa*. *Biol. Bull.* **145:** 140–149.

MCCLOSKEY, L. R., D. S. WETHEY, AND J. W. PORTER. 1978. The measurement and interpretation of photosynthesis and respiration in reef corals. Pp. 379–396 in D. R. Stoddart and R. E. Johannes, Ed., *Coral reefs: research methods*. UNESCO, Paris.

MUSCATINE, L. 1973. Nutrition of corals. Pp. 77–115 in Jones and Endean, Eds. *Biology and geology of coral reefs*. Vol. II. Academic Press, New York.

MUSCATINE, L., AND J. W. PORTER. 1977. Reef corals: mutualistic symbiosis adapted to nutrient-poor environments. *Bio-Science* **27:** 454–460.

PORTER, J. W. 1976. Autotrophy, heterotrophy and resource partitioning in Caribbean reef-building corals. *Am. Nat.* **110:** 731–742.

SIEGEL, S. 1956. *Nonparametric Statistics*. McGraw-Hill Book Co., New York. 312 pp.

SPENCER-DAVIES, P. 1977. Carbon budgets and vertical zonation of Atlantic reef corals. Pp. 391–396 in D. L. Taylor, Ed., *Proceedings Third International Coral Reef Symposium*, Vol. 1. University of Miami, Miami, FL.

SPENCER-DAVIES, P. 1980. Respiration in some Atlantic reef corals in relation to vertical distribution and growth form. *Biol. Bull.* **158:** 187–194.

Reference: *Biol. Bull.*, **160**: 303–310. (April, 1981)

THE ORIGIN OF THE NERVOUS SYSTEM IN *PENNARIA TIARELLA*, AS REVEALED BY TREATMENT WITH COLCHICINE*

VICKI JOAN MARTIN[1] AND MARY BETH THOMAS[2]

Wake Forest University, Winston-Salem, NC 27109

ABSTRACT

Treatment of 8-h embryos of the marine hydrozoan *Pennaria tiarella* with colchicine for 2 h eliminates interstitial cells, nematoblasts, and ganglionic cells. Comparisons of interstitial-cell-less and control planulae of *Pennaria* by TEM and light microscopy suggest a dual origin of the nervous system in *Pennaria*. Ganglionic cells are shown to be derived exclusively from endodermally-derived interstitial cells, whereas neurosensory cells are derived from cells of the ectodermal epithelium, probably from the epitheliomuscle cells. This investigation supports the idea that ganglionic cells and neurosensory cells can differentiate not only from different stem cells but also from different germ layers.

INTRODUCTION

Cnidarians are considered to be the first multicellular organisms in which a nervous system evolved (Davis, 1973). The origin of the various types of nerve cells in the cnidarians, namely, neurosensory, ganglionic, and neurosecretory, has been described in detail for *Hydra*. Generally, it is believed that all nerve cells arise from an embryonic reserve cell, the interstitial cell (McConnell, 1932; Burnett and Diehl, 1964; Lentz, 1965; Davis, 1969, 1972). The origin of nerve cells in cnidarians other than *Hydra* has not been reported.

A recent ultrastructural analysis of the nerve elements of the planula of the marine hydrozoan *Pennaria tiarella* (Martin and Thomas, 1980) identified two types of nerve cells. Type I nerve cells are at the base of the ectodermal epithelium just apical to the forming foot processes of the epitheliomuscle cells. Neurites projecting from these Type I nerve cells form a nerve plexus of transversely and longitudinally oriented processes. These Type I nerve cells resemble the ganglionic and sensory-motor-interneurons described for *Hydra* (Davis, 1971; Westfall, 1973). Type II nerve cells are restricted to the epidermis and are morphologically similar to the neurosensory cell described by Davis (1969) for *Hydra*.

The present study examined the origin of the nervous system in *Pennaria*. Colchicine was employed during embryonic development to produce planulae devoid of interstitial cells. These planulae were then examined for nerve cells by light and electron microscopy.

MATERIALS AND METHODS

Mature colonies of *Pennaria tiarella* were collected in August 1979 from wharf pilings in Morehead City, North Carolina. Fronds from male and female colonies

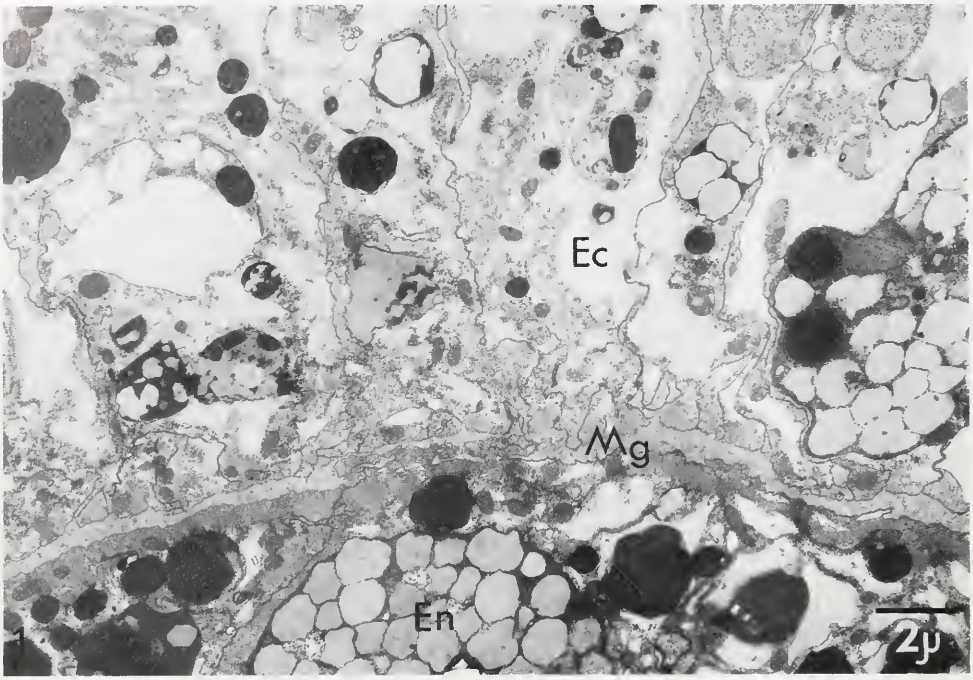
---

Received 9 September 1980, accepted 12 January 1981.

* Aided by a grant from Sigma Xi.

[1] Present Address: Department of Zoology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9.

[2] Present Address: Department of Biology, University of North Carolina at Charlotte, Charlotte, N. C. 28223.



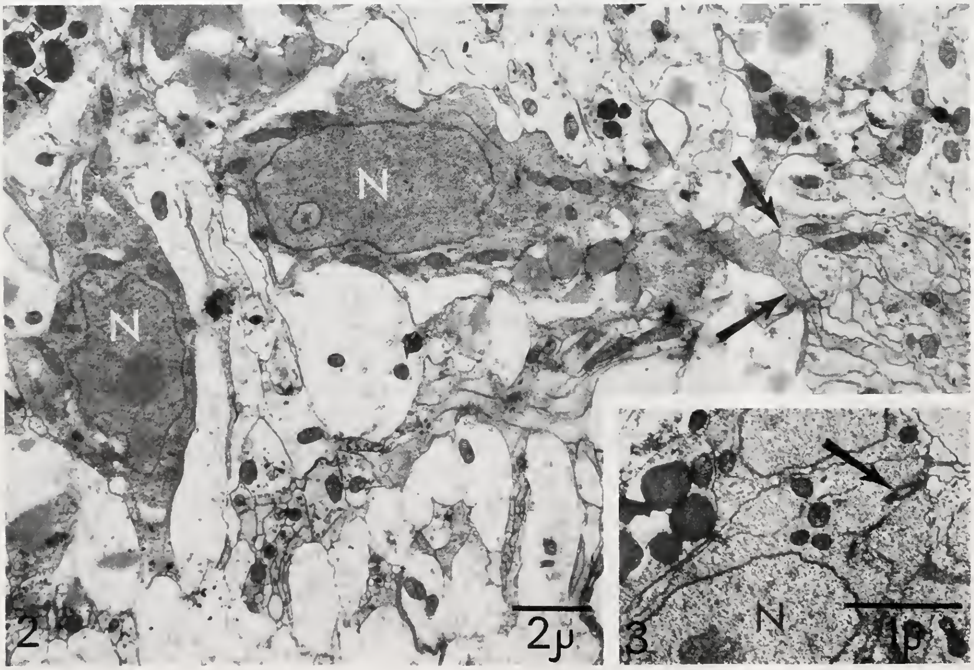


FIGURE 1. Transverse section of a colchicine-treated planula which has recovered for 72 h. Ganglionic cells are absent in these larvae. Ec = ectoderm; En = endoderm; Mg = mesoglea. Bar = 2 μm.

FIGURE 2. Transverse section of a region of a control planula comparable to the region of the colchicine-treated planula in Figure 1. Neurites (arrows) radiate out from the ganglionic cells, which occur between the developing foot processes of the epitheliomuscle cells and the nuclei of the ectodermal

were placed together at 5:00 p.m. in large finger bowls of filtered seawater. The bowls were placed in the dark at 6:00 p.m. At 9:00 p.m. early cleavage stages were removed to filtered seawater until 8 h after fertilization (3:00 a.m.).

The 8-h embryos were treated for 2 h in 0.4% colchicine (Sigma Corporation) dissolved in filtered seawater. The embryos then were washed repeatedly and placed in small finger bowls of filtered seawater. After a 12-, 24-, or 72-h recovery period in reagent-free normal seawater, experimental and control planulae were prepared for transmission electron microscopy (TEM) and light microscopy. Untreated and treated planulae were also examined at 48 h after fertilization.

Planulae to be examined by TEM were fixed for 1 h at room temperature in 2% glutaraldehyde in 0.1 *M* sodium-cacodylate-buffered seawater, pH 7, containing 0.044 *M* sucrose and 0.01 *M* $CaCl_2$ (modified from Anderson and Personne, 1970). The planulae were rinsed in three changes of this vehicle, for 10 min each, at room temperature. They were then postfixed in 1% osmium tetroxide in 0.1 *M* sodium cacodylate, pH 7, for 1 h and rinsed 30 min in the above buffer. The specimens were dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in an Epon-Araldite mixture. Specimens so fixed were sectioned with a diamond knife (MJO Company) on a Porter Blum MT2-B ultramicrotome. Sections were placed on copper grids, stained in 3% uranyl acetate (Watson, 1958) followed by 0.2% lead citrate (Veneable and Coggeshall, 1965), and examined with a Zeiss EM9S-2 electron microscope.

Plastic sections 0.18–0.36 μm thick from a region of the block adjacent to that used for thin sections were mounted on glass slides and stained with 0.5% toluidine blue in 1% sodium borate for light microscopy.

Planulae were fixed in 10% formalin in seawater and embedded in Paraplast Plus paraffin. Serial sections 10 μm thick were mounted on glass slides and stained with Azure B for detecting ribonucleic acids (RNA) (Swift, 1955). The cellular compositions of control and treated planulae were determined by counting the number of nuclei in individual cells from serial transverse sections of 82-h planulae. In both control and colchicine-treated planulae, 2000 cells were counted in each of 10 planulae. The number of epitheliomuscle or neurosensory cells (indistinguishable with the method used), mucous cells, interstitial cells, nematoblasts, and ganglionic cells per 20,000 cells was counted for each group. Gastrodermal epithelial cells were not counted.

RESULTS

The 82-h control planula of *Pennaria* displayed an array of highly organized cell types. The ectoderm was composed of epitheliomuscle cells, mucous cells, interstitial cells, nematoblasts, ganglionic cells, and neurosensory cells. The epitheliomuscle cells and mucous cells were abundant by 8 h after fertilization, constituting the majority of the cells in the ectoderm. Interstitial cells and a few forming nematoblasts first appeared in the endoderm between 8–10 h postfertilization. The interstitial cells and the nematoblasts increased in numbers as development proceeded. They eventually migrated to the ectoderm. Ganglionic cells arose at 24 h, whereas neurosensory cells were not present until 48 h after fertilization.

---

cells, and form a nerve plexus throughout the length of the planula. N = nucleus of ganglionic cell. Bar = 2 μm.

FIGURE 3. Ciliary rootlet (arrow) of a ganglionic cell. N = nucleus of ganglionic cell. Bar = 1 μm.

TABLE I

Average cell compositions of treated planulae of Pennaria tiarella *(percent of cells) after 72-h recovery. (20,000 cells counted per group.)*

Chemical Treatment	Epitheliomuscle Cells or Neurosensory Cells	Mucous Cells	Interstitial Cells	Nematoblasts	Ganglionic Cells
Control	52	17	10	12	9
Colchicine (0.4%)	80	20	0	0	0

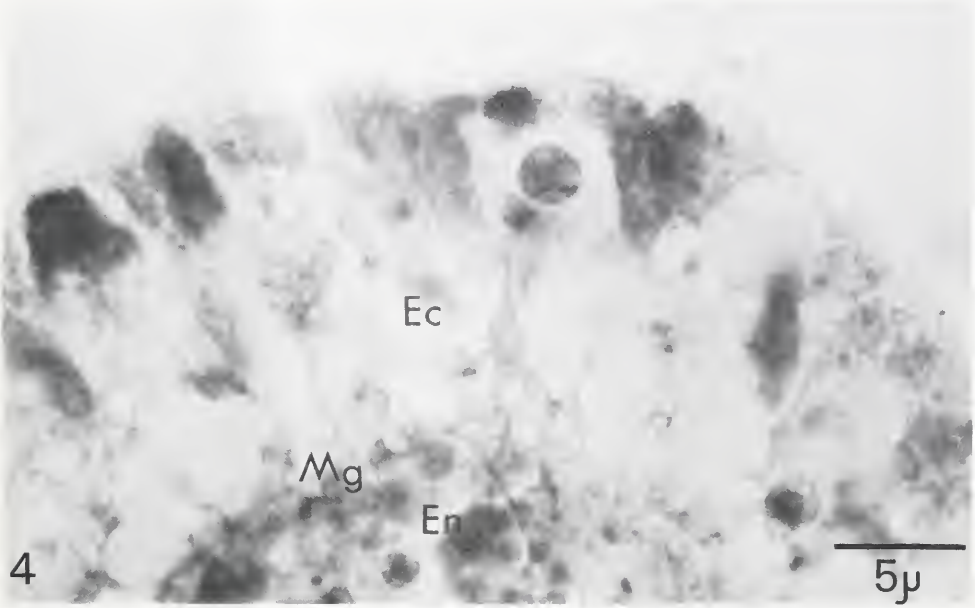
After examining, by light microscopy or TEM, approximately 70 embryos allowed to recover for 12, 24, or 72 h after treatment, we concluded that treatment of 8-h embryos for 2 h with colchicine completely eliminated interstitial cells, nematoblasts, and ganglionic cells (Table I). The elimination of these cells began approximately 22 h after fertilization and was completed by 34 h after fertilization. Table I shows the cellular compositions of control and treated planulae allowed to recover for 72 h (82-h planulae). The numbers are the percentage of each cell type relative to total cells counted. In controls, the interstitial cells, nematoblasts, and ganglionic cells comprised 31% of all cells present. Treatment with colchicine eliminated these cells, and only epithelial cells were found. Ultrastructural examination of these planulae (Fig. 1) repeatedly showed no ganglionic cells. In untreated planulae of comparable age, ganglionic cells and their corresponding neurites were abundant apical to the forming foot processes of the epitheliomuscle cells (Fig. 2). These ganglionic cells possessed a single cilium (Fig. 3) and contained neurosecretory droplets.

Ganglionic cells stain intensely with Azure B (Martin and Thomas, 1980). These Azure B-positive cells were diffusely distributed along the entire length of the larva, and corresponded to the neurite-producing cells seen by transmission electron microscopy. In colchicine-treated planulae the ganglionic cells were absent from transverse serial sections of paraffin-embedded material stained for RNA (Fig. 4). Azure B-positive ganglionic cells were present in corresponding controls (Fig. 5).

We observed neurosensory cells identical to those of control planulae in all the colchicine-treated larvae (Fig. 6). These cells appeared in the ectoderm 48 h after fertilization both in treated planulae devoid of interstitial cells and in control planulae. The cells extended from the free surface of the planula to the mesoglea. They were distinguished from epitheliomuscle cells and mucous cells in that they possessed a single cilium with a complex basal body assembly (Figs. 6 and 7) and numerous microtubules in the surrounding cytoplasm (Fig. 8).

DISCUSSION

Most studies on interstitial cells have been restricted to the freshwater genus *Hydra* (David and Campbell, 1972; David, 1973; Campbell and David, 1974; Marcum and Campbell, 1978). These cells are in the ectoderm of *Hydra* and presumably are the stem cells for all of the animal's nerve elements, nematocytes, and gametes (David and Gierer, 1974). Marcum and Campbell (1978) suggest that since these differentiated and specialized cells do not divide, elimination of the interstitial cells should result in the complete absence of nerve cells and other interstitial cell progeny.



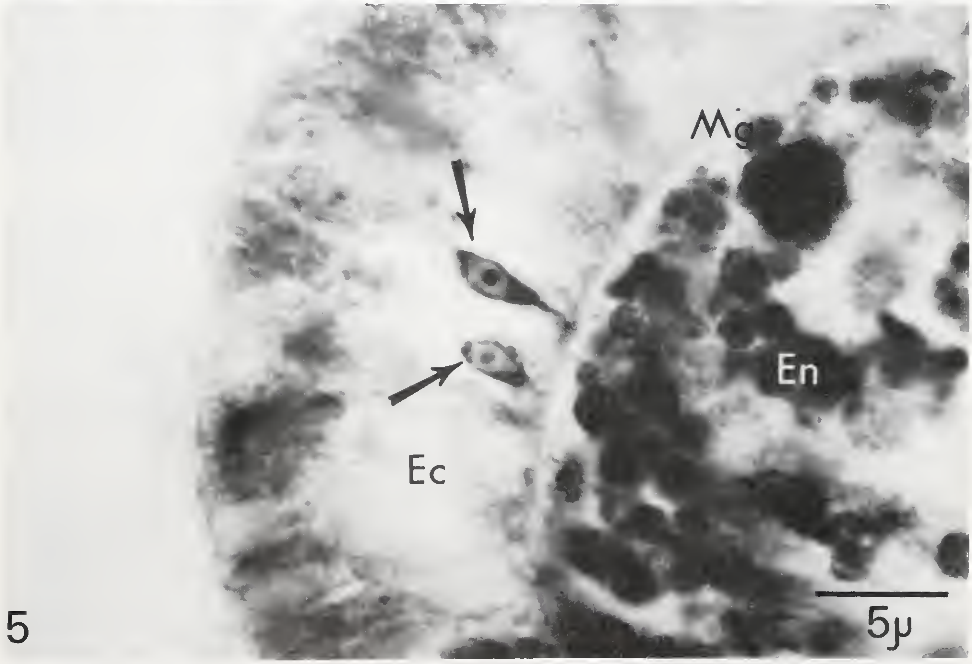


FIGURE 4. Light micrograph of a colchicine-treated planula (stained with Azure B) which has recovered for 72 h. Note the absence of ganglionic cells just apical to the mesoglea. Ec = ectoderm; En = endoderm; Mg = mesoglea. Bar = 5 μm.

FIGURE 5. Light micrograph of a control planula taken from a region comparable to that of Figure 4. Azure B-positive ganglionic cells (arrows) are in an area just above the mesoglea. Note the intense staining of the nucleolus and cytoplasm of these cells. Ec = ectoderm; En = endoderm; Mg = mesoglea. Bar = 5 μm.

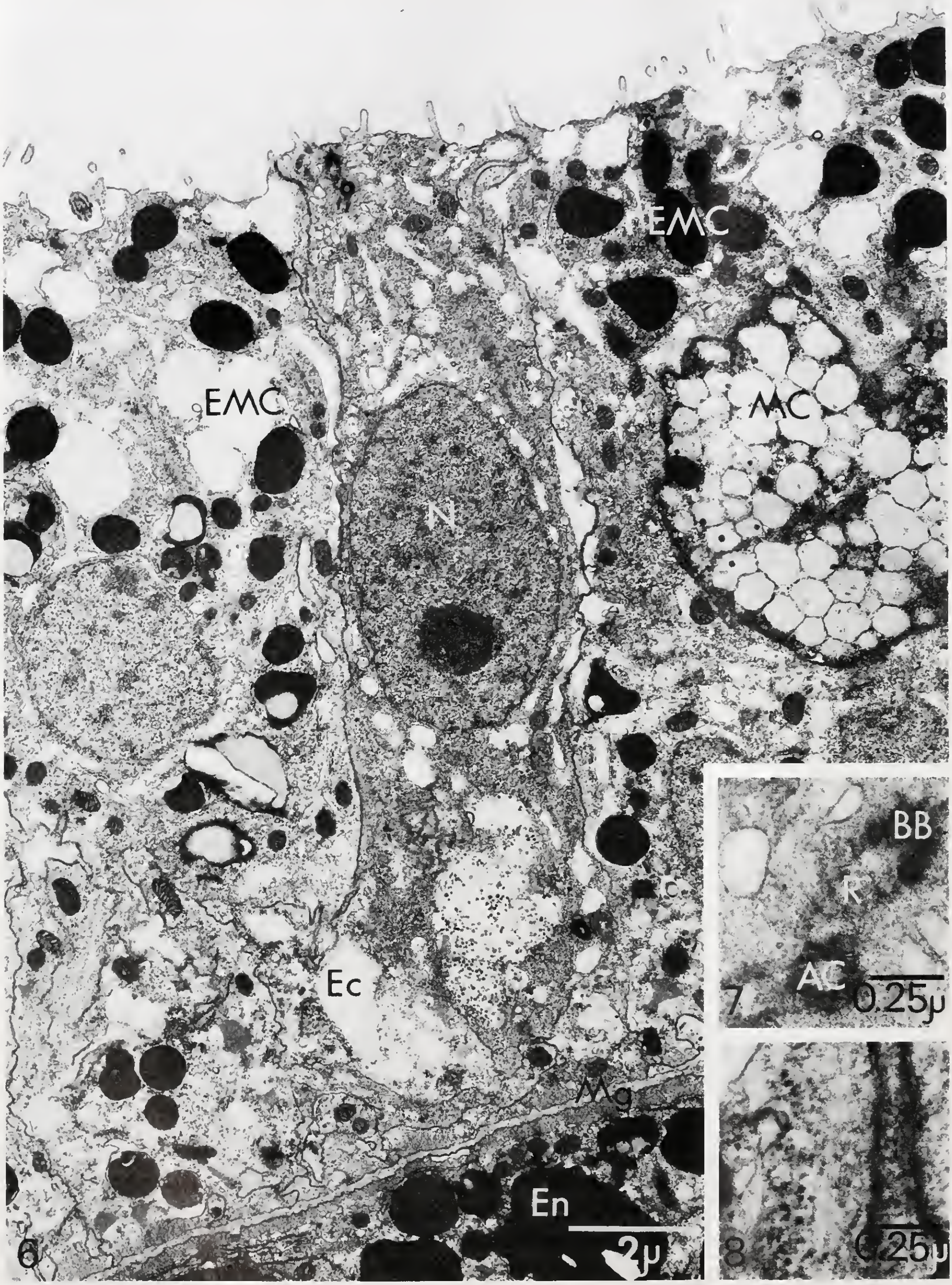


FIGURE 6. Neurosensory cell of a colchicine-treated planula which has recovered for 72 h after treatment. The cell is between two epitheliomuscle cells. Its morphology is identical to that of a neurosensory cell in a control planula. The cell extends from the free surface of the planula to the mesoglea and is characterized by a single cilium with a complex basal body assembly. Ec = ectoderm; EMC = epitheliomuscle cell; En = endoderm; MC = mucous cell; Mg = mesoglea; N = nucleus of neurosensory cell. Bar = 2 μm.

Campbell (1976) and Marcum and Campbell (1978) eliminated interstitial cells from *Hydra* by treating *Hydra* specimens for 8 h in a 0.4% solution of the alkaloid colchicine. The resultant *Hydra* specimens were devoid of interstitial cells, nerve cells, and nematocytes. However, they continued to bud, exhibit tissue renewal patterns, regenerate, and preserve polarity.

Numerous investigators have tried but failed to eliminate interstitial cells from various marine cnidarians (Campbell, personal communication). The elimination of the interstitial cells in 8-h embryos of *Pennaria tiarella* is to our knowledge the first such successful attempt.

In *Pennaria tiarella*, interstitial cells arise 8 h after fertilization from a central core of endodermal cells (Summers and Haynes, 1969; Martin and Thomas, 1977). Summers and Haynes (1969) report that these cells migrate to the ectoderm, where they differentiate into nerves and nematoblasts. Thus they suggest an endodermally-derived nervous system. Two distinct types of nerve cells have been identified with certainty in *Pennaria* (Martin and Thomas, 1980). The present study suggests a dual origin of the nervous system in *Pennaria*. When endodermally-derived interstitial cells were eliminated by colchicine, ganglionic cells were absent, but neurosensory cells were present. Furthermore, these neurosensory cells appeared 48 h postfertilization in planulae that had been rendered free of interstitial cells by 34 h postfertilization. If the interstitial cells were the sole stem cells for the nerve elements, then one would have expected that both ganglionic cells and neurosensory cells would have been eliminated. Thus, the neurosensory cells do not arise from interstitial cells.

We previously reported (Martin and Thomas, 1977) two types of ectodermally-derived cells in *Pennaria*: epitheliomuscle cells and mucous cells. The neurosensory cells must be added to the list. The neurosensory cell arises either from a stem cell of ectodermal origin or from dedifferentiation of epitheliomuscle cells or mucous cells. As we have never seen such a stem cell in the ectoderm, the first possibility seems unlikely. We have, however, observed dividing epitheliomuscle cells that gave rise to mucous cells. If epitheliomuscle cells produce mucous cells, then perhaps they also give rise to the neurosensory cells.

Thus, although ganglionic cells are interstitial cell derivatives, neurosensory cells may arise during larval development in *Pennaria* from some cell altogether different from the interstitial cell. Demonstration of a dual origin of the nervous system, together with the development of a method of eliminating one of the two nerve cell types, opens new avenues for analyzing cell function and intercellular interactions.

LITERATURE CITED

Anderson, W. A., and P. Personne. 1970. The localization of glycogen in the spermatozoa of various invertebrate and vertebrate species. *J. Cell Biol.* **44:** 29–51.

Burnett, A. L., and N. A. Diehl. 1964. The nervous system of *Hydra*. I. Types, distribution, and origin of nerve elements. *J. Exp. Zool.* **157:** 217–226.

Campbell, R. D. 1976. Elimination of *Hydra* interstitial and nerve cells by means of colchicine. *J. Cell Sci.* **21:** 1–13.

Figure 7. Basal body (BB), accessory centriole (AC), and rootlet (R) of a neurosensory cell from a colchicine-treated planula. Bar = 0.25 μm.

Figure 8. Microtubules in the apical cytoplasm of a neurosensory cell from a colchicine-treated planula. Bar = 0.25 μm.

Campbell, R. D., and C. N. David. 1974. Cell cycle kinetics and development of *Hydra attenuata.* II. Interstitial cells. *J. Cell Sci.* **16:** 349–358.

David, C. N. 1973. A quantitative method for maceration of *Hydra* tissue. *Wilhelm Roux Archiv. Entwichlungsmech. Org.* **171:** 259–268.

David, C. N., and R. D. Campbell. 1972. Cell cycle kinetics and development of *Hydra attenuata.* I. Epithelial cells. *J. Cell Sci.* **11:** 557–568.

David, C. N., and A. Gierer. 1974. Cell cycle kinetics and development of *Hydra attenuata.* III. Nerve and nematocyte differentiation. *J. Cell Sci.* **16:** 359–375.

Davis, L. E. 1969. Differentiation of neurosensory cells in *Hydra. J. Cell Sci.* **5:** 699–726.

Davis, L. E. 1971. Differentiation of ganglionic cells in *Hydra. J. Exp. Zool.* **176:** 107–128.

Davis, L. E. 1972. Ultrastructural evidence for the presence of nerve cells in the gastrodermis of *Hydra. Z. Zellforsch.* **123:** 1–17.

Davis, L. E. 1973. Ultrastructure of neurosensory cell development. P. 271 in A. L. Burnett, Ed., *Biology of Hydra.* Academic Press, New York.

Lentz, T. L. 1965. The fine-structure of the differentiating interstitial cells in *Hydra. Z. Zellforsch.* **67:** 547–560.

Marcum, B. A., and R. D. Campbell. 1978. Development of *Hydra* lacking nerve and interstitial cells. *J. Cell Sci.* **29:** 17–33.

Martin, V. J., and M. B. Thomas. 1977. A fine-structural study of embryonic and larval development in the gymnoblastic hydroid, *Pennaria tiarella. Biol. Bull.* **153:** 198–218.

Martin, V. J., and M. B. Thomas. 1980. Nerve elements in the planula of the hydrozoan *Pennaria tiarella. J. Morphol.* **166:** 27–36.

McConnell, C. H. 1932. The development of the ectodermal nerve net in the buds of *Hydra. Q. J. Microsc. Sci.* **75:** 495–509.

Summers, R. G., and J. F. Haynes. 1969. The ontogeny of interstitial cells in *Pennaria tiarella. J. Morphol.* **129:** 81–88.

Swift, H. 1955. Cytochemical methods for nucleic acids. P. 91 in E. Chargaff and J. H. Davidson, Eds., *The Nucleic Acids.* Academic Press, New York.

Veneable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25:** 407–408.

Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.* **4:** 475–479.

Westfall, J. A. 1973. Ultrastructural evidence for a granule-containing sensory-motor-interneuron in *Hydra littoralis. J. Ultrastruct. Res.* **42:** 268–282.

Reference: *Biol. Bull.*, **160**: 311–321. (April, 1981)

ADAPTIVE SIGNIFICANCE OF A SEMILUNAR RHYTHM IN THE TERRESTRIAL CRAB *SESARMA*

MASAYUKI SAIGUSA

Okayama University, College of Liberal Arts, Department of Biology,
Tsushima-Naka 2-1-1, Okayama, 700 Japan

ABSTRACT

Larval release activities of the terrestrial crab *Sesarma* were observed 1.5 km upriver from the sea. The number of *Sesarma haematocheir* and *Sesarma intermedium* females releasing larvae peaked twice monthly, during the full and new moon periods. Larval release, at about dusk, coincided with high water at the nearby seacoast. Larvae of *S. haematocheir* and *S. intermedium* died quickly in fresh water. The semilunar rhythm of larval release gives the larvae, released just after high water of spring tides and around high water on the days following the full and new moons, a better chance of reaching the sea than otherwise. *Sesarma dehaani* did not reveal a clear semilunar rhythm and the time of day of larval release did not coincide well with high water. *S. dehaani* inhabits riverbanks and rice paddies near the sea, and its larvae have the highest tolerance to fresh water. These factors may account for its lack of synchronization with tides.

INTRODUCTION

A number of marine organisms exhibit lunar or semilunar rhythms in reproductive activities (Korringa, 1947; Enright, 1975; Naylor, 1976). Several recent works, particularly on the polychaete *Platynereis* (Hauenschild, 1960), the intertidal midge *Clunio* (Neumann, 1966, 1975, 1976, 1978) and the terrestrial crab *Sesarma* (Saigusa, 1980a) studied these rhythms as biological clocks.

The adaptive advantages of fewer of these reproductive rhythms have been reported. Since almost all known lunar and semilunar rhythms are reproductive, it is presumed that they enhance synchronization of the sexes for mating. For species with external fertilization, restricting the release of gametes to a particular time and place enhances the possibility of successful fertilization (Klapow, 1972).

Further insight into reproductive synchrony may be sought for species with internal fertilization. Oka and Hashimoto (1959) reported that the intertidal midge, *Clunio tsushimensis*, emerges at the time of morning low water for a few days before spring tides. According to Neumann (1966), emergence of *Clunio marinus* is synchronized with the time of afternoon low water of spring tides. This synchronization of emergence would assure both mating and suitable substrata for the succeeding egg-laying.

Clunio deposits fertilized eggs onto the exposed substrata. Many other organisms with lunar or semilunar rhythms release larvae. (For example, several *Uca* species have been reported to exhibit semilunar rhythms of reproduction; Christy, 1978; Zucker, 1978.) When such animals inhabit estuarine flats or banks where tidal excursions are fairly large, families might have to release their larvae during the most favorable tides. Therefore, the sexes might have to synchronize mating (Naylor, 1976), or females might have to synchronize egg-laying (*i.e.*, attachment of fertilized eggs to the ovigerous hairs).

Received 26 July 1979, accepted 5 December 1980.

Grapsid crabs of the genus *Sesarma* are mainly found on and near the estuarine shores. They inhabit riverside banks near the sea. Among them, *Sesarma (Holometopus) haematocheir* (de Haan), *Sesarma (Sesarma) intermedium* (de Haan), and *Sesarma (Holometopus) dehaani* H. Milne Edwards are land dwellers inhabiting hillsides and rice paddies beyond estuarine shores (Saigusa, 1978). From June to September, ovigerous females of these species move to the riverside or seashore and release their larvae (zoeae) into water. The author has already reported a semilunar rhythm of larval release in *S. haematocheir* and *S. intermedium*, and briefly referred to the adaptive significance of this rhythm (Saigusa and Hidaka, 1978). The present paper confirms the semilunar rhythm of larval release in these two species on the basis of data obtained in 1975, and further reports a larval release rhythm of *S. dehaani*. In addition, this paper presents evidence indicating that the phase relationship between larval release rhythm and the semilunar cycle of tides is an adaptation to help zoeae released into a stream reach the sea as soon as possible.

MATERIALS AND METHODS

Observations were carried out from June–September 1975 and from 10 July–24 September 1976 on the riverside in the southern end of Izu Peninsula, about 1.5 km upriver from the sea (Saigusa, 1980b). The author sat on a rock close to the stream every evening and counted the number of females that released larvae into the stream. With the aid of an electric torch (4.8 V midget lamp), counting was done in a 5-m reach along the water's edge. Despite daytime searches many times from June to July 1975, almost every day from 1 July to 13 August 1976, and sometimes after that I never observed release of larvae during daytime. All-night observations 9–10 June and 22–23 July 1975, and 28–29 August 1976, made it clear that females did not release larvae in the early morning. Therefore, except on the days just cited, counting started about 30 min before sunset every day and finished around 24:00 at the latest in 1975 and around 1:30 at the latest in 1976. Further details and behavior of ovigerous females upon larval release have been described elsewhere (Saigusa, 1980b; Saigusa and Hidaka, 1978).

The following experiments were intended to determine the tolerance of zoeae to fresh and sea water. Sixteen test tubes (1.7 cm diameter and 15 cm depth, or 3 cm diameter and 20 cm depth) were prepared for each experiment. Ovigerous females of *Sesarma haematocheir, S. intermedium,* and *S. dehaani* were captured on the hillsides fronting Gokasho Bay of Kii Peninsula in 1979 and brought into the laboratory at Kyoto. They were kept in plastic containers. Zoeae released into fresh water at the bottom of the containers were transferred into the test tubes. Three individual zoeae were placed in each test tube and the surviving individuals were counted at intervals of 2–24 h. Spring water (chlorinity 0.02‰; chlorinity = grams chloride ion in 1 kg H_2O) and city water (chlorinity 0.04‰, kept standing for a day) were used as fresh water. Sea water from Gokasho Bay of Kii Peninsula was used after rough filtration. Further experiments examined tolerance of zoeae of *S. haematocheir* and *S. intermedium* to sea water diluted with fresh water in the following ratio: 1:20 sea water and 1:10 sea water. All these experiments were carried out at room temperature.

The time needed for zoeae released at the observation site (Izu Peninsula) to reach the sea was estimated by floating corks down the river. A small lead was suspended from each cork by a string 10–20 cm long. Five corks were thrown into the river at the observation site 1 h after high water of spring tides (at 6:15 on 28

May 1979) and at low water (at 12:00 on 27 May 1979), respectively. When stagnant pools or dead trees, etc., captured corks, they were picked up and rethrown into the middle of the stream, or new corks were thrown in.

RESULTS

At the observation site, the river was about 7–10 m wide. It was a shallow stream about 20 cm deep at low water, rising about 50–100 cm at high water and on rainy days. Since I saw no zoeae released into tributaries, I concluded that females inhabiting hillsides along this river move to the main stream. Figure 1 shows a chlorinity gradient in the river at high water and low water on 24 August 1980 (2 days before the full moon). At high-water periods, tidal influence reached 1.7 km upriver from the sea, but chlorinity was not different from fresh water more than 1.25 km upriver from the sea (0.03–0.04‰).

From mid-June to September, ovigerous females of *Sesarma dehaani* moved to the riverside and released larvae into water. *S. intermedium* females released larvae from late June to September, *S. haematocheir* from early July to September.

Figures 2A and B show the observed number of females releasing zoeae every night for *S. haematocheir* and *S. intermedium.* (Since no data were collected for several days between 21 July and 8 August, data obtained in 1976 for corresponding lunar phases were substituted.) Zoea-release activities in *S. haematocheir* and *S. intermedium* peaked twice monthly during both the full and new moon periods—thus, they had a semilunar rhythm. The number of *S. intermedium* females releasing zoeae during the full moon was about the same as the number during the new moon (Fig. 2B). This was also seen in 1976. More *S. haematocheir* females were seen during the full moon than during the new moon (Fig. 2A). The populations observed in 1976 also demonstrated the full moon periods for larval release activities.

Figure 2C shows the number of *S. dehaani* females observed releasing larvae every night from 21 June to 9 July and from 21 July to 8 August 1975, and from 10 July to 13 September 1976. The number of females revealed no concentration of larval release activities in *S. dehaani* about each full and new moon.

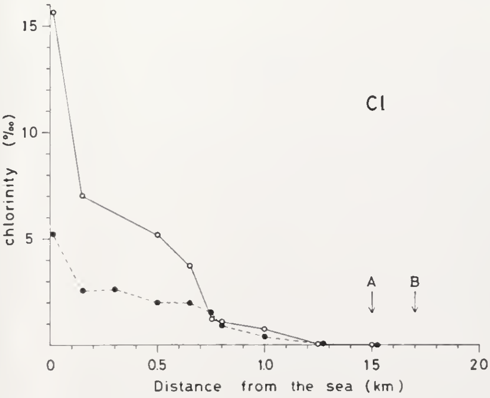
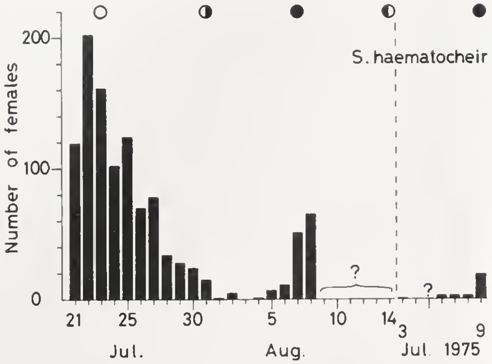


FIGURE 1. Chlorinity (content of chloride ion in NaCl) gradient in the river during low water (broken line) and high water (solid line). Water was sampled from 9:50 to 10:35 and 17:00 to 17:40 on 24 August 1980 (2 days before the full moon). Chlorinity was calculated by titration with $AgNO_3$. Arrows show the locations of the observation site (*A*) and the end of tidal influence (*B*).



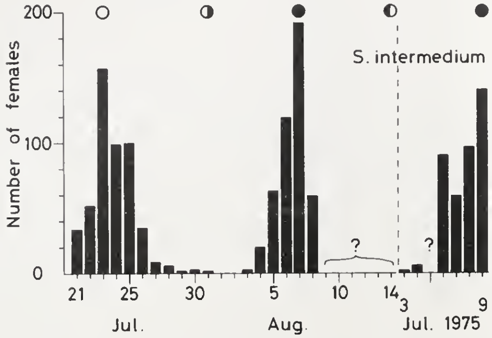


FIGURE 2A. (Above) Number of *Sesarma haematocheir* releasing larvae per night 21 July–8 August and 3 July–9 July 1975. Data from 25, 27, 29, and 31 July and 1 and 3 August replaced by data obtained on 10, 12, 14, 16, 17, and 19 September 1976, respectively; each lunar phase of the former days was identical with the lunar phase of the latter days.

FIGURE 2B. (Below) Number of *Sesarma intermedium* releasing larvae per night 21 July–8 August and 3 July–9 July 1975. For days when observations were not carried out, data obtained on 10, 12, 14, 16, 17, and 19 September 1976 (identical lunar phases) were substituted.

Figure 3 shows zoea-release by each of the three species. All these species released their larvae between sunset and midnight. Zoea-release activities in both *S. haematocheir* and *S. intermedium* females were highly synchronized and timed to coincide with lunar and tidal phase. Around the full and new moons, zoea-release activities in these two species was concentrated just after sunset. On the days following the full and new moons, zoea-release took place in accordance with the time of high water occurring at the nearby seacoast or the time of moonrise or moonset. Around the first and last quarters of the moon, few females released zoeae, and zoea-release occurred sporadically in the early evening. As shown in Figures 3A and B, almost all larvae were released into the stream within 2.5 h after afternoon high water. In 1975, *S. dehaani* females also showed a weak zoea-release rhythm, with a 15-day period coinciding with the lunar phase but only slight synchronization with high water. Semilunar synchronization was more distinct in *S. haematocheir* and *S. intermedium*, which inhabit higher land than *S. dehaani*.

The survival curves of zoeae are shown in Figures 4 and 5. Fresh water severely affected *S. haematocheir* and *S. intermedium* zoeae. Within 6 h of treatment, 5–15% of *S. haematocheir* zoeae died. All died within 48 h. Within 4 h of treatment,

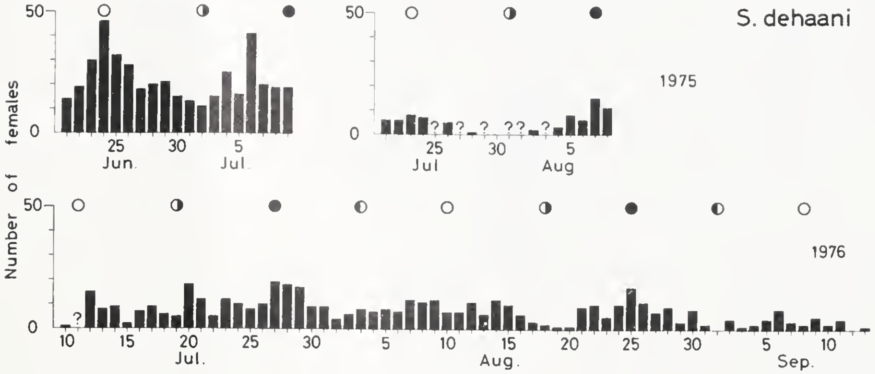


FIGURE 2C. Number of *Sesarma dehaani* releasing larvae per night 21 June–9 July and 21 July–8 August 1975; and 10 July–13 September 1976. Observations not carried out on 26, 27, and 30 June and 1, 2, and 5 July 1975. Each day of no observations was substituted by superposing data of 2 days examined in 1976; phase of larval release rhythm can be regarded as identical (26 June replaced by 14 and 28 July; 27 June by 15 and 29 July; 30 June by 18 July and 1 August; 1 July by 19 July and 2 August; 2 July by 20 July and 3 August; and 5 July by 23 July and 6 August 1976, respectively. See Fig. 3C).

5–45% of *S. intermedium* zoeae died and all died within 70 h. *S. dehaani* zoeae had the highest tolerance of fresh water among the three species. However, a dead zoea was found 17 h after the treatment and all died within 96 h. Survival times grew longer as salinity increased (Fig. 5). Zoeae placed in natural sea water all lived at least 4 days, except one *S. haematocheir* zoea. Thus, zoeae released in fresh water are exposed to immediate danger of death. This may be particularly true for zoeae of *S. haematocheir* and *S. intermedium*.

During low water, the river ran very slowly. Mud flats, stones, and dead trees were exposed to the air. Stagnant pools were common, and soon collected all corks; new or re-thrown corks soon entered another pool. At 150 m from the sea, they were borne upriver by the rising tide. However, during high water, stagnant pools were scarce. Two corks took 3 h 20 min and 3 h 30 min, respectively, to reach the sea with the receding tide, though they stopped at the water's edge several times.

DISCUSSION

At the observation site, zoeae were released in fresh water (chlorinity 0.03–0.04‰). As Figure 4 shows, their survival would be immediately threatened. The capture of corks released at low water, and the successful journey to the sea of two corks thrown into the stream just after high water, may apply to zoeae released at the observation site. Zoeae released during low water would soon get into stagnant pools. It would be very difficult for them to reach the sea or even brackish water until the following high water, receding, carries them downriver. On the other hand, zoeae released at high water may successfully reach the sea with the receding tide. High and low waters occur at intervals of about 6.2 h. Corks took about 3.5 h to reach the sea. Therefore, zoeae released within about 2.7 h after high water would have a chance of reaching the sea with the receding tide.

Figure 3 shows that almost all zoeae of *S. haematocheir* and *S. intermedium* were released into the stream within 2.5 h of high tide. They thus might successfully reach the sea with the receding tide. Larvae released just after the time of high

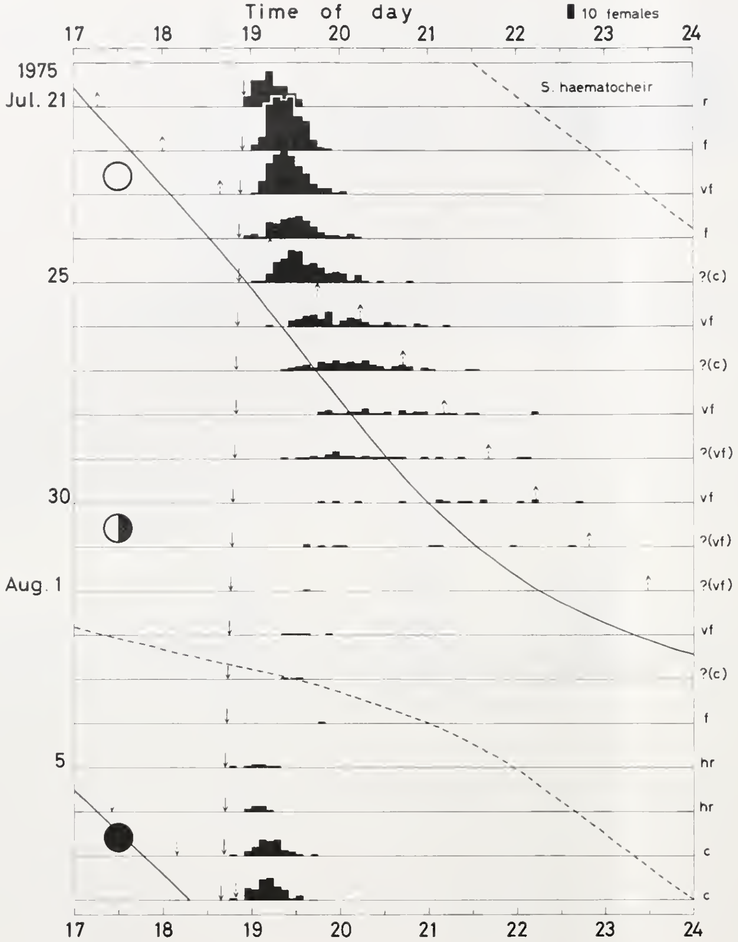


FIGURE 3A. Time of day of larval release in *Sesarma haematocheir* 21 July to 8 August 1975, shown by number of females observed per 5 min in relation to lunar phase, times of high (solid diagonal lines) and low (broken diagonal lines) tides occurring at the nearby seacoast, times of sunset (downward solid arrows) and times of moonrise (upward broken arrows) and moonset (downward broken arrows). Weather during observations: very fine (vf), fine (f), cloudy (c), rain (r), heavy rain (hr). As explained in Fig. 2A, substitute data was used for field observations not carried out on 25, 27, 29, 31 July and 1 and 3 August. Sunset times also substituted on these days.

water of spring tides might have the best chance of reaching the sea and larvae released at the time of high water on the following days would have a better chance of reaching the sea than during other tidal phases.

Larval release activities in *S. dehaani* showed no clear semilunar rhythm (Fig. 2C), and were not well synchronized or timed to coincide with high tides occurring after sunset (Fig. 3C). The zoeae of this species have the highest tolerance of fresh water among three species. Even released at ebb tide, they would live in fresh water until the following receding water carried them downriver. In addition, distribution of this species was limited to bottomlands near the sea (Saigusa, 1978). Many crabs were found in the riverside banks and rice paddies downstream from the observation site. Thus, females may release their larvae into brackish water.

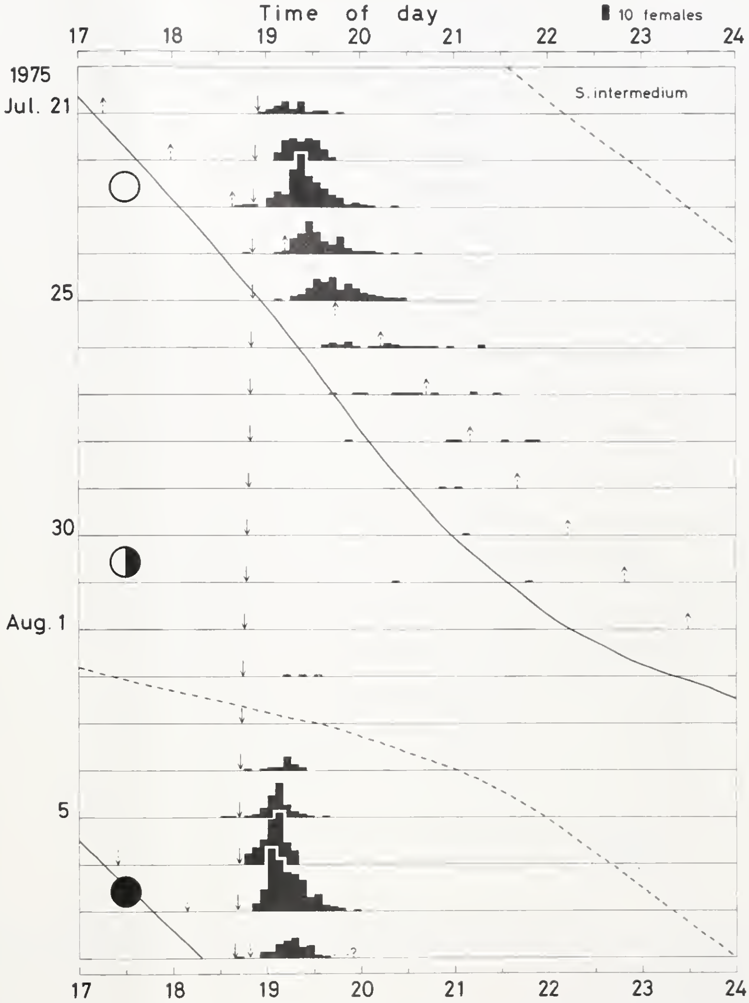


FIGURE 3B. Time of day of larval release in *Sesarma intermedium*, 21 July–8 August 1975. Symbols as in Fig. 3A. For days when observations were not carried out, treatment was as in Fig. 3A.

Larval release in *S. haematocheir* and *S. intermedium* peaked around the full and new moons (Figs. 2A, B). As reported elsewhere (Saigusa and Hidaka, 1978), peaks were often 1–5 days after the full and new moons, at the time of optimal tides (Figs. 3A, B). However, in a few cases the number of *S. intermedium* females releasing zoeae suddenly increased 3–5 days before the full moon: 5 and 7 August and 5 September 1976. Figure 6 shows a case (in September 1975) where larval release in *S. haematocheir* peaked 3 days before the full moon. In these cases, larvae were released at close the time of ebb tide or during the rising tide, making adaptive benefits hard to explain.

As shown in Figures 3A, B, and C, females of the three species released their larvae only in the early half in the night and not in the morning high-water period. One possible explanation is that timing is not determined by a requirement of the larvae, but rather by the females' requirement of moving to the place of larval

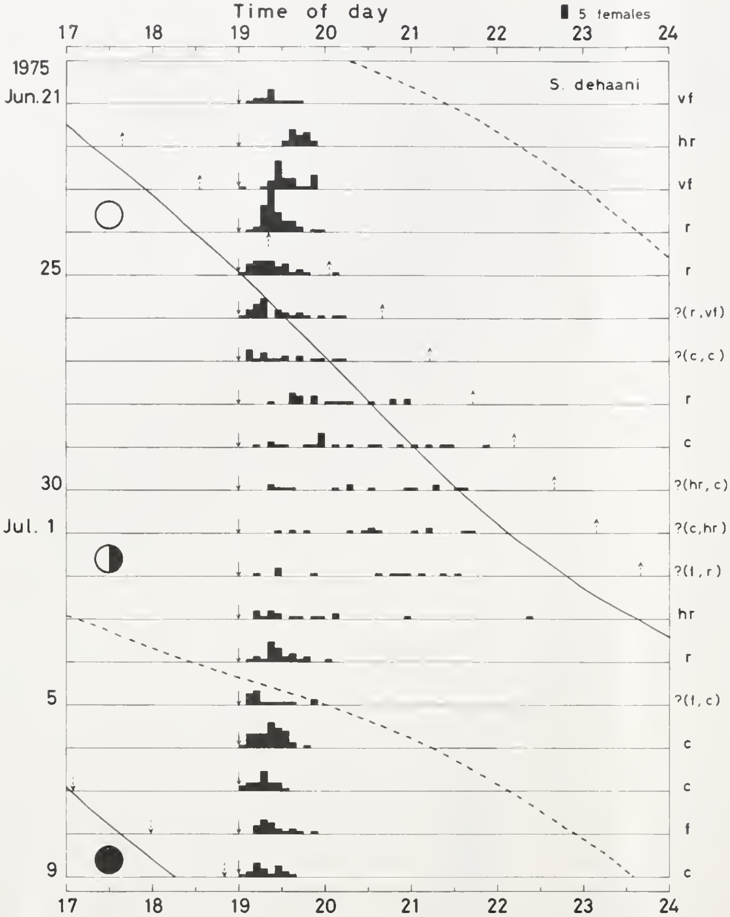


FIGURE 3C. Time of day of larval release in *Sesarma dehaani*, 21 June–9 July 1975. Two days when phase of larval release rhythm are regarded as identical were substituted for a day of no observations. Sunset times also substituted.

release. Ovigerous females move to the shore during the daytime. Around both the full and new moons they release larvae with the onset of night. On the following days, some wait until high water before releasing larvae.

On coasts with semidiurnal tides, though the times of high and low waters on days of full and new moons always occur at nearly the same time of day for years, they differ at various locations on the seashore. According to Neumann (1975, 1976, 1978), the time of emergence and subsequent reproduction was correlated with local low waters. The semidiurnal cycle of tides shows time displacements of a few hours along the coasts of Japan, especially in the Inland Sea. It is not known whether larval release activities in *S. haematocheir* and *S. intermedium* are synchronized with such local tidal conditions.

Further insight into adaptive benefits of a semilunar rhythm of larval release may be expected. Christy (1978) recently proposed a hypothesis to explain the semi-monthly release of *Uca pugilator* larvae. He estimated that juvenile crabs would be ready to settle on an intertidal flat about 3 weeks later, i.e., during spring

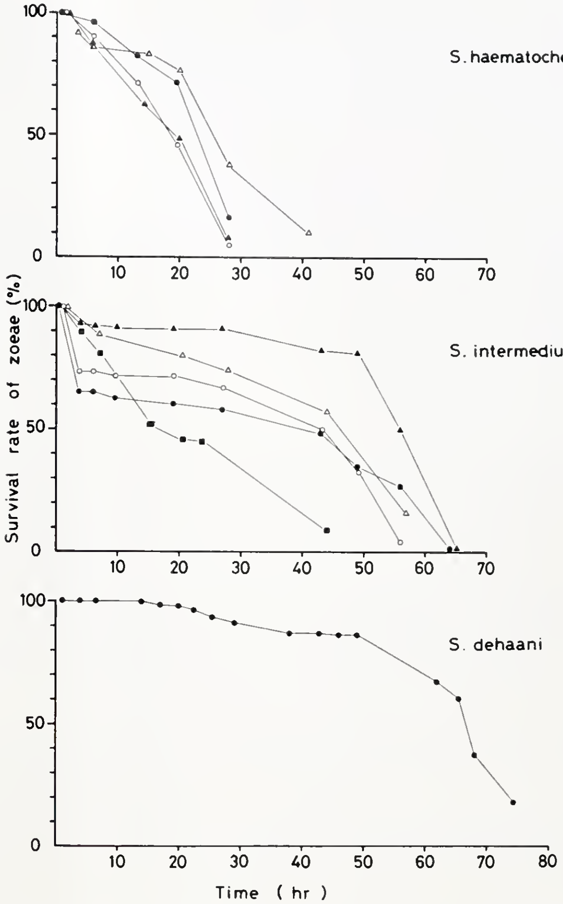


FIGURE 4. Survival of zoeae in fresh water. The ordinate indicates time after the release of zoeae.

tides, and demonstrated that spring-tide currents would help ensure up-estuary transport toward suitable adult habitats. Zucker (1978) suggested that the same might be true for the three tropical *Uca* species, though their courtship activities are displaced a week, from the neap-tide to the spring-tide period.

According to Baba and Miyata (1971), larvae of *S. dehaani* took at least 22 days to become young crabs, passing through four zoea and one megalopa stages. Hashimoto (1968) reported megalops of these three species in river mouths from July to October. This suggests that larvae of *S. haematocheir* and *S. intermedium* return to the river mouth about 1 month after they are released into the river. Megalops which return to the river mouth around spring tides would be able to swim to the most distant riverside from the sea with the rising tide. This might expand adult habitats.

Zoeae also are released in brackish and sea water (Saigusa, in preparation), by females inhabiting hillsides where there is no suitable river. According to Hashimoto (1965), in a few cases *S. haematocheir* females release their larvae into tributaries whose water level is not influenced by tides. In such cases, the semilunar rhythm of larval release may have no adaptive significance. However, a large num-

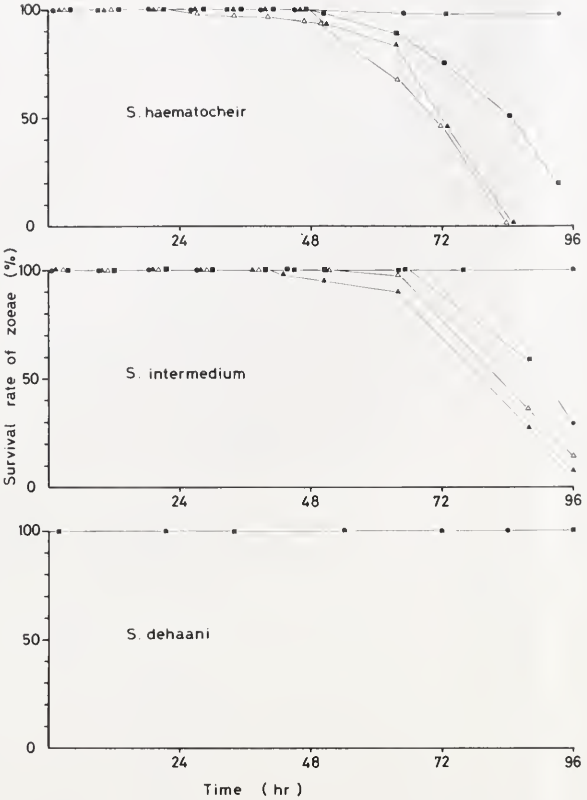


FIGURE 5. Survival of zoeae in natural sea water (closed circles) and diluted sea water (open and closed triangles, 1:20 sea water; closed square, 1:10 sea water).

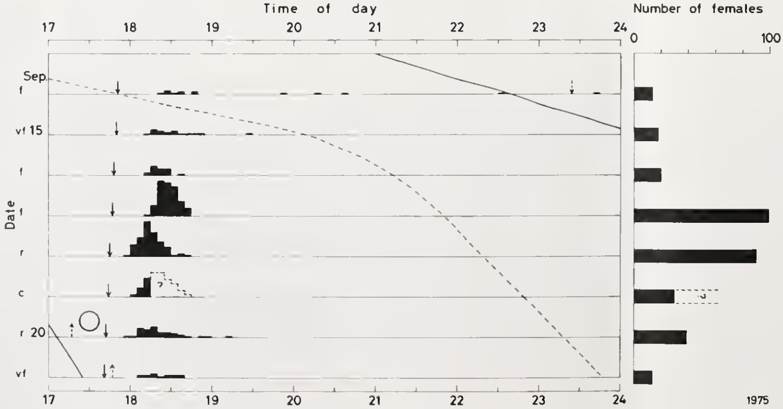


FIGURE 6. Number of *Sesarma haematocheir* females releasing larvae per night (right) and the time of day of release (left) 14 September–21 September 1975. High tide: solid slanted line; low tide: broken slanted line; sunset: downward solid arrow; moonset: downward broken arrow; weather very fine (vf), fine (f), cloudy (c), rain (r), heavy rain (hr).

ber of zoeae are usually released in fresh water; release into a relatively large stream like the one studied is common. Therefore, the semilunar rhythm by *S. haematocheir* and *S. intermedium* females may be regarded as important to survival of their larvae.

ACKNOWLEDGMENTS

I wish to thank Prof. Emeritus Masaru Kato, Kyoto University, and Prof. Hiroshi Watanabe, Director of Shimoda Marine Research Center, The University of Tsukuba, for kindly providing facilities for this work. Thanks go to Prof. Dietrich Neumann, University of Köln, who gave me many valuable suggestions on the present study and kindly read the manuscript. This is a contribution of the Shimoda Marine Research Center, University of Tsukuba.

LITERATURE CITED

BABA, K., AND K. MIYATA. 1971. Larval development of *Sesarma (Holometopus) dehaani* H. Milne Edwards (Crustacea, Brachyura) reared in the laboratory. *Mem. Fac. Educ., Kumamoto Univ.* **19,** Sec. 1 (Nat. Sci): 54–64.

CHRISTY, J. H. 1978. Adaptive significance of reproductive cycles in the fiddler crab *Uca pugilator*: a hypothesis. *Science* **199:** 453–455.

ENRIGHT, J. T. 1975. Orientation in time: endogenous clocks. Pp. 917–944 in O. Kinne, Ed., *Marine ecology, Vol. II, Physiological mechanisms, Part 2.* John Wiley & Sons, London.

HASHIMOTO, H. 1965. The spawning of the terrestrial grapsid crab, *Sesarma haematocheir* (DE HAAN) *Zool. Mag. (Tokyo)* **74:** 82–87 (In Japanese with English summary).

HASHIMOTO, H. 1968. Ecological distribution of the megalops of the terrestrial sesarmid crabs in the river. *Bull. Fac. Educ., Shizuoka Univ. Nat. Sci. Ser.* **19:** 55–63 (In Japanese with English summary).

HAUENSCHILD, C. 1960. Lunar periodicity. *Cold Spring Harbor Symp. Quant. Biol.* **25:** 491–497.

KLAPOW, L. A. 1972. Fortnightly molting and reproductive cycles in the sand-beach isopod, *Excirolana chiltoni. Biol. Bull.* **143:** 568–591.

KORRINGA, P. 1947. Relations between the moon and periodicity in the breeding of marine animals. *Ecol. Monogr.* **17:** 349–381.

NAYLOR, E. 1976. Rhythmic behaviour and reproduction in marine animals. Pp. 393–429 in R. C. Newell, Ed., *Adaptation to the environment: essays on the physiology of marine animals.* Butterworths, London.

NEUMANN, D. 1966. Die lunare und tägliche Schlüpfperiodik der Mücke *Clunio.* Steuerung und Abstimmung auf die Gezeitenperiodik. *Z. Vergl. Physiol.* **53:** 1–61.

NEUMANN, D. 1975. Lunar and tidal rhythms in development and reproduction of an intertidal organism. Pp. 451–463 in F. J. Vernberg, Ed., *Physiological adaptation to the environment.* Intext Educational Publishers, New York.

NEUMANN, D. 1976. Adaptations of chironomids to intertidal environments. *Ann. Rev. Entomol.* **21:** 387–414.

NEUMANN, D. 1978. Entrainment of a semilunar rhythm by simulated tidal cycles of mechanical disturbance. *J. Exp. Mar. Biol. Ecol.* **35:** 73–85.

OKA, H., AND H. HASHIMOTO. 1959. Lunare Periodizität in der Fortpflanzung einer pazifischen Art von *Clunio* (Diptera, Chironomidae). *Biol. Zentralbl.* **78:** 545–559.

SAIGUSA, M. 1978. Ecological distribution of three species of the genus *Sesarma* in winter season. *Zool. Mag. (Tokyo)* **87:** 142–150.

SAIGUSA, M. 1980a. Entrainment of a semilunar rhythm by a simulated moonlight cycle in the terrestrial crab, *Sesarma haematocheir. Oecologia (Berl.)* **46:** 38–44.

SAIGUSA, M. 1980b. Lunar and semilunar rhythms in reproduction. *Aquabiology* **2:** (I) 248–254, (II) 372–377 (In Japanese).

SAIGUSA, M., AND T. HIDAKA. 1978. Semilunar rhythm in the zoea-release activity of the land crabs *Sesarma. Oecologia (Berl.)* **37:** 163–176.

ZUCKER, N. 1978. Monthly reproductive cycles in three sympatric hood-building tropical fiddler crabs (genus *Uca*). *Biol. Bull.* **155:** 410–424.

Reference: *Biol. Bull.*, **160**: 322–331. (April, 1981)

GROWTH AND REPRODUCTION OF THE GIANT GLOSSIPHONIID LEECH *HAEMENTERIA GHILIANII*

ROY T. SAWYER[1], FRANCOIS LEPONT[2], DUNCAN K. STUART[1],
AND ANDREW P. KRAMER[1]

[1]*Department of Molecular Biology, University of California, Berkeley, CA 94720; and* [2]*Office de la Recherche Scientifique et Technique Outre-Mer Cayenne 973, French Guyana*

ABSTRACT

A continuously breeding laboratory colony of the giant leech *Haementeria ghilianii* has been established from a few specimens collected in French Guyana. The leeches feed on live rabbits or bovine blood, which they draw from an artifical feeding device. Leech growth is saltatory, in that at each of four successive feedings, spaced over about half a year, the weight of each specimen increases 3- to 6-fold. The male reproductive system of this hermaphroditic leech matures first, at a body weight of 3–5 g. The female reproductive system matures after at least one more feeding, at a body weight of at least 8 g. Fecundity (number of eggs per laying) depends on the weight of the leech at the time of oviposition, increasing from 60 for the smallest mature individuals to 500 for the largest. These data on the growth and reproduction of *H. ghilianii* gathered under laboratory conditions are consistent with its probable life cycle in the native habitat.

INTRODUCTION

In the latter part of the 19th century, C. O. Whitman, the first director of the Woods Hole Marine Biological Laboratory, pioneered the use of glossiphoniid leeches for the study of animal development (Whitman, 1878, 1887). Using such leeches, Whitman was the first to show that a definite developmental fate can be assigned to the descendants of identifiable cells of an early embryo. By the turn of the century, leeches had become objects of intense interest, not only to embryologists but also to the founders of modern neuroanatomy, who, in their efforts to correlate the structure of the nervous system with its function (Leydig, 1862; Retzius, 1891), were attracted to the relatively simple, readily accessible, and highly stereotyped leech nervous system.

Unaccountably, interest in leeches as materials for embryological and neurological studies declined in the first decades of this century. Neurological interest in leeches was revived only in the 1960s, when it was found that modern electrophysiological methods, such as the recording of nerve cell activity by means of intracellular electrodes, can be applied with great profit to the leech nervous system (Nicholls and Kuffler, 1964). Meanwhile, such neurobiological studies have provided far-reaching insights into the leech nervous system, particularly its identified nerve cells and their synaptic connections (Nicholls and Van Essen, 1974). Moreover, these studies have led to a neurological account of moderately complex behavioral routines, such as the leech swimming movement, in terms of networks of identified neurons (Stent *et al.*, 1978). In view of these recent advances, the time seemed favorable to resume the embryological investigation of leeches as well, particularly within the context of contemporary developmental neurobiology.

Received 14 October 1980, accepted 14 January 1981.

However, the European medicinal leech, *Hirudo medicinalis*, with which most of the recent neurobiological work has been carried out, is not a particularly favorable species for embryological studies. *H. medicinalis* belongs to the Hirudinidae, whose members lay very small eggs and are sustained by larval organs in their early development. However, the members of another family, the Glossiphoniidae (studied by Whitman), lay larger eggs and are sustained by their yolk (Schleip, 1936). Thus the glossiphoniid egg is a more favorable object for the study of leech development.

For this reason we sought a glossiphoniid species with the following desirable properties: (1) easy and continuous breeding under laboratory conditions; (2) large eggs amenable to embryological analysis and manipulation; and (3) a nervous system amenable to neurophysiological analysis. Adults of most members of the Glossiphoniidae are so small that they lack the third property. But in 1849, one specimen of an exceptionally large glossiphoniid, designated *Haementeria ghilianii*, was discovered near the mouth of the Amazon (Filippi, 1849a, b). The species' existence subsequently was confirmed both in Brazil (Lang, 1890) and in neighboring French Guyana (Blanchard, 1899). Adult specimens were reported to reach lengths of up to 50 cm in full extension. Hence it appeared that this giant tropical glossiphoniid, of which no report had been made since 1899, might possess all three of the desirable properties. Accordingly, we secured live specimens of *H. ghilianii* in French Guyana. With these specimens we established a continuously breeding colony in 1976 at the University of California at Berkeley (Sawyer, 1978). Studies carried out on embryos and adult specimens obtained from that colony have shown that *H. ghilianii* is indeed amenable to developmental and neurophysiological analysis.

This article reports observations on the feeding habits, dynamics of growth, and development of reproductive potential of *H. ghilianii* under laboratory conditions.

MATERIALS AND METHODS

The *H. ghilianii* founder specimens of the present laboratory colony were collected in a marsh near Sinnamary, French Guyana. The water of the native marsh of these specimens is acidic (pH 5.8–6.2), undergoes diurnal temperature variation from about 25°C–35°C, and has a low oxygen content, particularly at the bottom. Being near the equator, the marsh has about 12 h of daylight throughout the year.

To approximate these conditions in the laboratory, the leeches are maintained in shallow, 2.6 m × 1.2 m basins filled to a depth of about 5 cm with deionized water to which is added 0.63 m*M* NaCl, 0.07 m*M* $CaCl_2$, 0.07 m*M* $MgCl_2$, and 0.01 m*M* K_2SO_4, to provide cations in their approximate natural concentrations. Buffering at pH 5.9 is provided by adding 1 m*M* morpholine ethane sulfonic acid (MES). The water is filtered or changed periodically to prevent waste products, such as ammonia from leech urine, from rising to toxic levels. After feeding, it is essential to change the water daily for several weeks. The water temperature is 25°C–27°C. The basins are in a greenhouse where the midday natural light intensity (measured at the water surface) varies from about 150 lux (on a cloudy day) to more than 500 lux (on a sunny day). In addition, incandescent light at an intensity of 100 lux at the water surface is provided year-round from 0600 to 1800 h.

Under our laboratory conditions *H. ghilianii* has been found to feed on the blood of mammals (cattle, rabbit, rat, mouse), less readily on reptiles (turtles), and not at all on fish (goldfish) or amphibia (frog). To withdraw blood from its mammalian or reptilian host, the leech attaches itself to the host's skin by means of its anterior sucker, and inserts its proboscis hypodermically. For economical laboratory feeding of adult *H. ghilianii*, we devised an artificial blood feeding device: This is a 23-cm-long plexiglass box with two compartments, each 10 cm in width, separated by a freshly prepared piece of skin from a cow's head. The compartment faced by the inner surface of the cowskin is filled to a depth of about 10 cm with fresh, heparinized (6 mg/l heparin salt) bovine blood. The other compartment, faced by the outer, hairy surface of the skin, is filled to the same depth with water. Hungry leeches placed in the water compartment attach themselves to the piece of skin, pierce it, and take in blood. Upon withdrawal of the proboscis after feeding, the small hole pierced in the skin by the proboscis closes, so there is little leakage between the two compartments.

The artificial feeding device is not used for the first blood meal of juvenile leeches, since a juvenile could find the cow skin too thick for its smaller proboscis. Hence, for the first feeding, the brooding parent leech, to whose venter the juveniles are still attached, is placed in an aquarium with an adult rabbit, whose ventral fur has been shaved. The aquarium is filled with enough water to cover the rabbit's venter. Under these conditions, juveniles ready for their first meal leave the parent, attach themselves to the rabbit, and draw its blood while they feed, later returning to the parent.

RESULTS

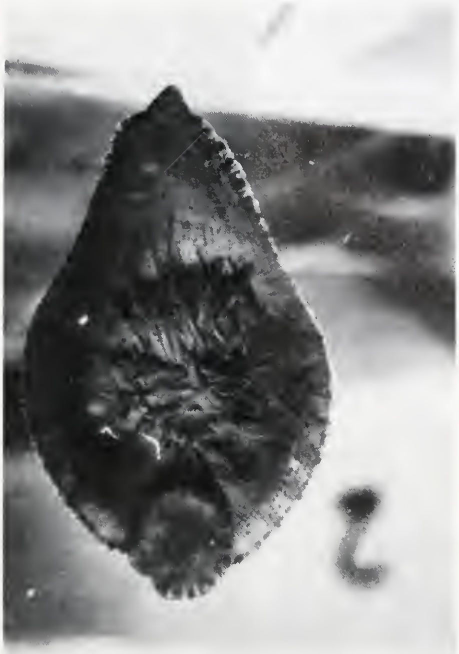
Reproduction

H. ghilianii, like all leeches, is a protandrous hermaphrodite. To mate, the sperm donor leech brings its male gonopore into contact with the body of another leech and ejaculates one spermatophore, which becomes implanted in the body wall of the recipient. Fertilized eggs issue from the female pore enclosed in a membranous sac, or cocoon, which remains attached to the ventral body wall of the brooding parent. Embryonic development of the fertilized egg begins upon oviposition and proceeds directly, via a series of highly stereotyped cleavages, to the formation of a juvenile leech attached by its posterior sucker to the venter of the brooding parent (Fig. 1). The embryonic development of *H. ghilianii*, which will be described in detail elsewhere, generally is similar to that previously reported for other glossiphoniid leeches (Schleip, 1936), especially for *Helobdella triserialis* (Weisblat *et al.*, 1978, 1980) and *Theromyzon rude* (Fernandez, 1980; Fernandez and Stent, 1980). The egg of *H. ghilianii* is 2.0–2.5 mm in diameter, of density 1.15 gm/cm^3, and weighs 5–8 mg. It is rich in yolk, which sustains the embryo until, as a juvenile, it has become capable of feeding. Individual *H. ghilianii* juveniles shortly before feeding weigh 10–18 mg, or about twice as much as the eggs from which they developed. Most of that increase in weight must come from water assimilated into body tissues during embryogenesis.

Dynamics of growth

To ascertain when the leeches are ready to take their first meal, *i.e.*, reach the status of juveniles, a cohort of 366 hatchlings brooded by a single parent was given the opportunity to feed on a rabbit about once a week. Of the cohort 19% took their first meal on the 27th day after oviposition and 67% on the 33rd day. All but four of the remaining juveniles took their first meal on the 40th day.

To ascertain the trophic effects of feedings, the growth of eight juveniles was



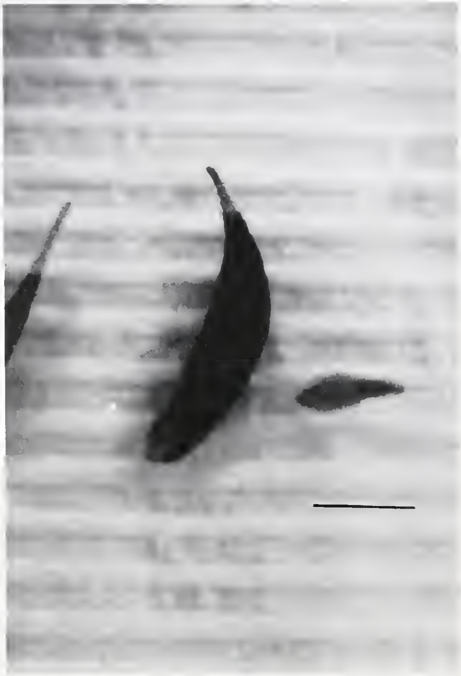


FIGURE 1. (Left) *H. ghilianii* juveniles attached to the venter of their parent. The length of this specimen is about 10 cm from head (top) to the circular caudal sucker (bottom). (Photograph courtesy of Timothy Branning.)

FIGURE 2. (Right) *H. ghilianii* juveniles just before (right) and just after (center and left) their first feeding. Bar: 1 cm.

followed through sexual maturity. At first feeding, of about 20 min, the juveniles ingested about 6 times their pre-feeding body weight in blood (Fig. 2). The animals would not feed again for about a month, presumably until the ingested blood had been metabolized. The long-term increase in body weight resulting from the first feeding approximately equaled the amount of blood ingested. About a third of the weight of ingested blood was eliminated as urine within a first few days after feeding (*cf.* Table I), some fraction of the first meal probably was released as CO_2 and feces, and the remainder of the long-term weight increase must have reflected assimilation of water into the body tissues.

At their second feeding (Table I) of about an hour, the leeches ingested about 5 times their body weight in blood. During the ensuing 2 months, digestion and assimilation of the second meal again resulted in a long-term increase in weight about equal to that of the ingested blood. At the third feeding, the blood intake was again about 5 times the body weight, resulting in an approximately equal increase in long-term body weight during the following 3 months. Finally, at the fourth feeding, which lasted nearly 2 h, the blood intake was about 3 times the pre-feeding weight. Digestion of this fourth meal required 3 months or more.

Thus, as shown in Figure 3, the post-embryonic growth of *H. ghilianii* is best described as saltatory. The relative size of each step remained roughly constant but the duration increased progressively. However, among individuals of the same cohort the parameters of this saltatory development varied considerably—partic-

TABLE I

Weight changes associated with successive feedings of H. ghilianii. *Data are mean values of measurements made on 8 isolated specimens of* H. ghilianii *whose individual growth was monitored from juvenile to sexually mature adult. All four feedings were on a rabbit host.*

Number of Feeding	First	Second	Third	Fourth
Days since first feeding	0	36	92	180
Feeding time (minutes)	22	56	—	110
Body weight before feeding (grams)	0.017	0.11	0.74	4.6
Blood ingested (grams)	0.093	0.54	3.4	14.2
Relative increase in body weight during feeding	640%	580%	560%	400%
Ingested blood retained after urine elimination (grams)	0.063	0.44	2.7	11.8
Long-term increase in weight of body tissues due to feeding (grams)	0.096	0.63	3.9	11.4

ularly the amount of blood ingested per feeding, and hence the increase in body weight. In the eight leeches followed individually for securing the data of Table I, this variation resulted in a distribution of individual weights after the fourth feeding so broad that its standard deviation was about half the mean.

Under our laboratory conditions, the leeches live 1 to 2½ years: Of mature individuals (including the eight specimens reported in Table I and Figure 3), five died between the ages of 12 and 18 months, two died between the ages of 18 and 24 months, and the remaining four died between the ages of 24 and 29 months.

Maturation

The development and maturation of the male and female reproductive systems of *H. ghilianii* was related to body weight, and hence to the number of meals the animal had taken. The male reproductive system matured first at body weights of about 3–5 g. (Spermatophore discharge was not observed from individuals of lesser weight, whereas such discharges were common in the 3–5 g weight range.) By contrast, the female reproductive system did not normally mature until at least one more feeding, when body weight exceeded 10 g (Fig. 6). Some individuals laid eggs after only three feedings, but these already weighed 8–16 g.

Some time intervenes between the feeding that enables the female reproductive system to mature, and egg-laying. During that time the ingested blood is presumably digested and assimilated. Thus, of 129 individuals that became gravid during 1 year, none had fed within 2 months, 25% had fed within 3 months, 75% had fed within 5 months, and all had fed within 7 months. The time elapsed between feeding and oviposition appears to depend on such variables as the age and physiological state of the individual at the time of feeding and its weight after feeding. As shown in Table II, even among a cohort of six leeches, the time between the fourth feeding and egg-laying ranged from 2–5 months. Some leeches lay a second clutch of eggs as early as 2 months after the first clutch, without an intervening feeding or mating.

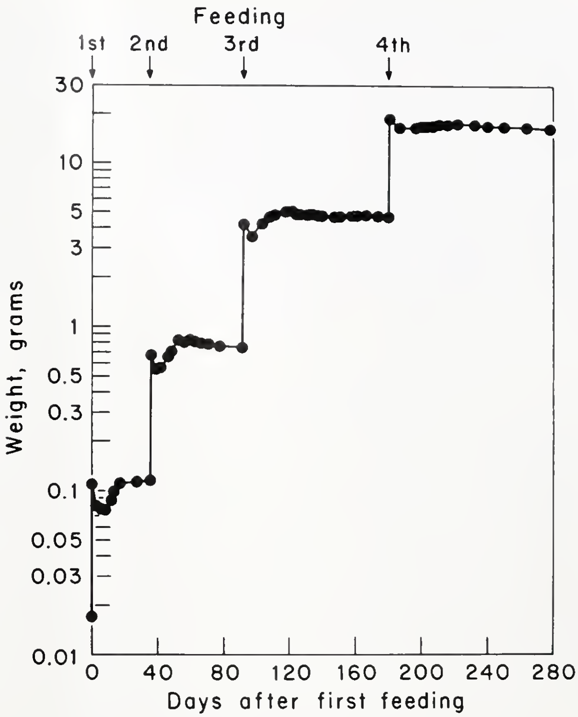


FIGURE 3. Growth dynamics of *H. ghilianii*, from juvenile to sexually mature adult, from mean weight of a cohort of eight isolated specimens provided four feedings on a rabbit host. The loss of weight in the first few days after feeding is presumably attributable to the elimination of fluid as urine.

After the female reproductive system matures, the male system retains its capacity to implant spermatophores. To ascertain where the donor leech implants its spermatophores on the recipient, we surveyed the location of spermatophores on the bodies of reproductively mature specimens. This survey showed (Fig. 4) that donors implant spermatophores onto all parts of the recipient's dorsal and ventral body surface except the head, and most commonly on the dorsolateral surface of

TABLE II

Time between feeding and egg-laying: Six H. ghilianii *specimens belonging to a cohort that had its third feeding 8 months earlier were given their fourth feeding on the same rabbit. Individual leeches laid eggs after the number of days shown had elapsed. The eggs were removed and counted, and the leech was then weighed.*

Leech Number	Days Between Feeding and Egg-Laying	Number of Eggs Laid	Weight of Leech (g)
1	58	149	16.0
2	59	275	24.8
3	65	272	23.5
4	75	232	22.3
5	108	246	21.5
6	152	321	30.5

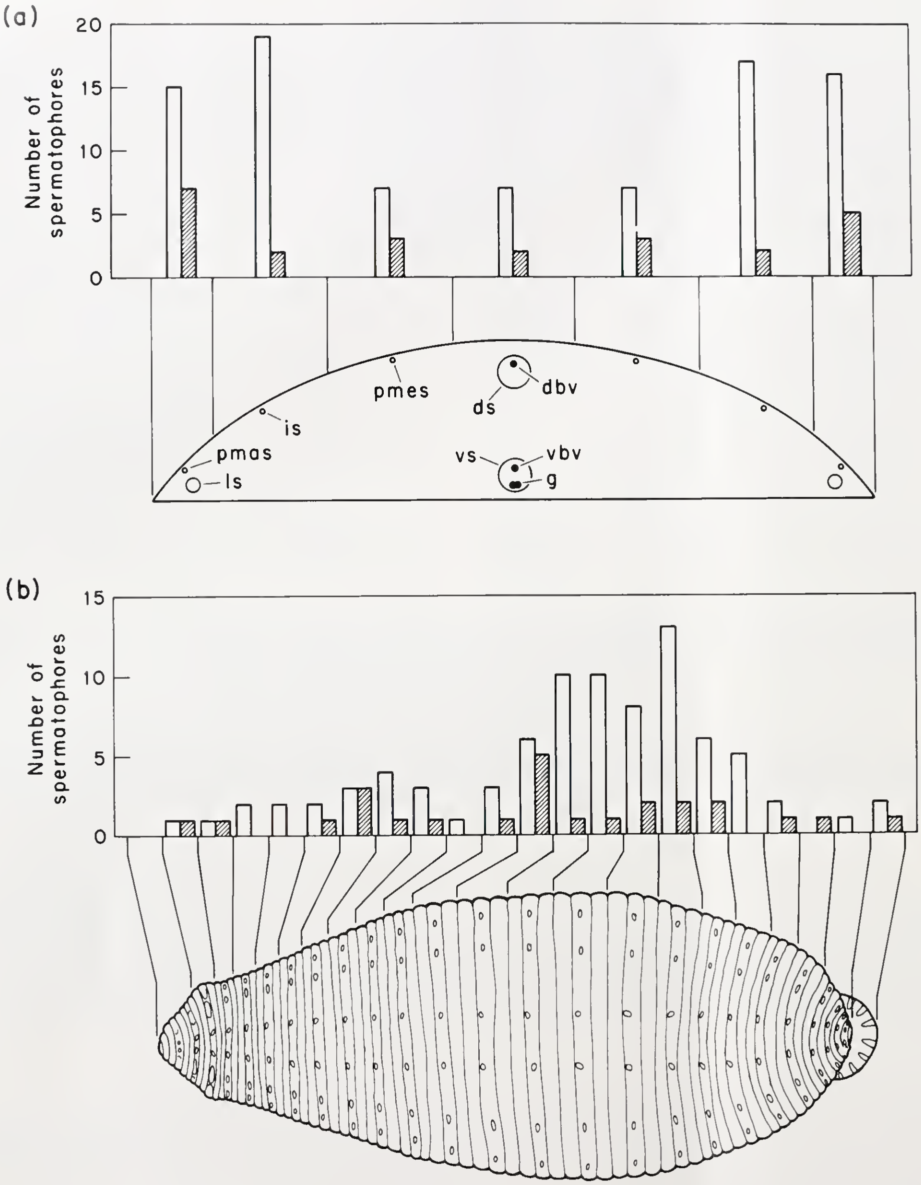


FIGURE 4. Distribution of implanted spermatophores over the body surface of a population of *H. ghilianii* adults. Open or crosshatched bars indicate the number of spermatophores found on the dorsal or ventral surface, respectively, within the domain delimited by the lines drawn to the body plan shown below the histogram. (a) Mediolateral distribution over 7 arbitrary sectors of the transverse crossection. dbv = dorsal blood vessel; ds = dorsal sinus; g = ganglion; is = intermediate sinus; ls = lateral sinus; pmas = paramarginal sinus; pmes = paramedial sinus; vbv = ventral blood vessel; vs = ventral sinus. (b) Rostrocaudal distribution over the body segments shown at the bottom. Open circles represent sensillae marking the central annulus of each body segment.

the posterior body segments. This concurs with Myers' (1935) survey of spermatophore-implantation sites on *H. parasitica.* These preferred sites of implantation are close to the sinuses of those segments in which the eggs are carried

(Mann, 1962), thus minimizing the distance that the sperm released from the implanted spermatophore have to migrate to reach the eggs.

Gravid leeches could be recognized some weeks before they laid eggs, by a whitish clitellum that appeared in the ventral region surrounding the gonopores. Closer to the time of egg-laying, a large yellow area, showing yolky eggs in the ovisacs, could be seen on the ventral midline posterior to the gonopores. About 2 weeks before leeches laid eggs, the ventral inflection of the lateral margins of the body gradually increased. This inflection eventually produced a brood pouch that protected the eggs in their cocoons attached to the venter of the parent (Fig. 5). To ensure fertilization of their eggs under the conditions of laboratory culture, gravid individuals were placed in a separate container with a consort of about 6 active sperm-donor individuals.

The cocoons were normally deposited in pairs, one on each side of the ventral midline. When the number of cocoons formed was uneven, one unpaired cocoon usually took a medial position at the front. Figure 6, the results of a study of the fecundity of 125 *H. ghilianii* individuals, shows that the number of eggs per individual per laying increased with body weight, from about 100 for the smallest leeches with mature female reproductive systems (10 g) to about 350 for the largest leeches (50 g). (One exceptionally large, 80 g leech laid nearly 500 eggs.) Table II also shows that the number of eggs laid increased with body weight. In contrast, the number of cocoons per individual showed no significant relation to body weight; the number varied between 6 and 12, with a typical value of 10. Thus, the increase with maternal weight in egg number per individual was accompanied by an increase in mean number of eggs per cocoon per individual, from a low of about 10 for the 10 g individuals to a high of about 35 for the 50 g individuals.

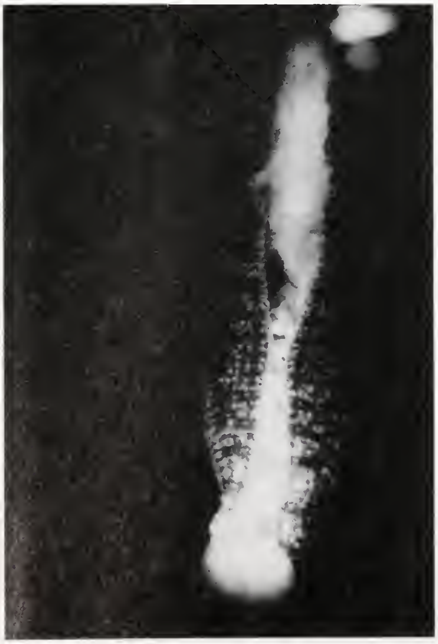


FIGURE 5. *H. ghilianii* brood pouch, in which cocoons (with their eggs and early embryos) are held.

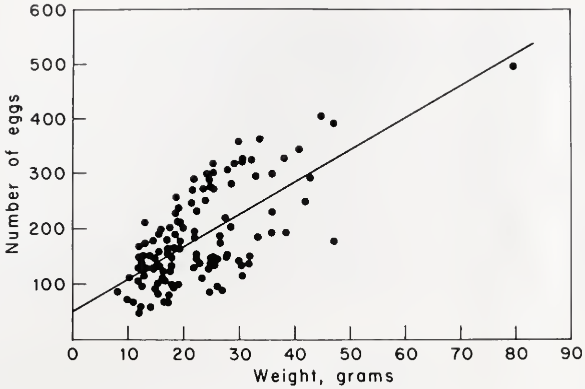


FIGURE 6. Fecundity of leeches as a function of body weight in terms of the number of eggs laid per individual. Each point represents the oviposition of one leech of weight shown on the abscissa. The straight line is a least-squares fit to the data points.

DISCUSSION

The data presented here on the laboratory rearing of *H. ghilianii* provide some insights into the life cycle of the leech in its native French Guyana habitat. In accord with the saltatory post-embryonic growth dynamics of laboratory-reared *H. ghilianii* (Fig. 3), the natural population in the marsh where the colony originated consisted of apparent weight classes, corresponding to laboratory-reared individuals after one, or two, or more feedings (Sawyer and LePont, unpublished observations). Similarly, Herter (1936) reported that natural populations of the glossiphoniid leech *Theromyzon tessulatum* comprise specimens falling into four distinct size classes, and inferred from this that leeches' mode of growth is "fractionated." One critical ecological factor in the *H. ghilianii* life-cycle is the dry season, from September to November. During that season the marsh's water level falls and daytime water temperature rises (up to 34°C at the surface). The leeches avoid these adverse conditions by remaining burrowed in the bottom mud, surviving by metabolizing previously ingested blood. Upon return of the rains in December, presumably much of the population is hungry; leeches emerge from the bottom mud to take another meal (Sawyer and LePont, unpublished observations). Thus we can expect that many reproductively mature individuals will feed synchronously after the dry season. Since the time of egg-laying is critically dependent on when the leeches feed, we predict that a wave of egg-laying occurs in the population after the dry season. On the basis of our laboratory data (Table II), this egg-laying wave would occur 2–7 months after the dry season ends, or normally in February to July.

ACKNOWLEDGMENTS

We thank Ian Madin for innovative contributions to this project, Milan Tomic, Brian Jennison, and Mooyung Choi for maintenance of the leech colony, and Margery Hoogs and Gunther Stent for helpful discussions and suggestions for the preparation of the manuscript. This work was supported by NIH Research Grant HL 22227; NIH Postdoctoral Fellowship NS 05996; NSF Postdoctoral Fellowship SPI-79L4849; a Postdoctoral Fellowship from the Miller Institute for Basic Research in Science; and a grant from the Penrose Fund of the American Philosophical Society.

LITERATURE CITED

Blanchard, E. 1899. Courtes notices sur les Hirudinées. XXVIII. Sur de *Liostomum Ghilianii* (F. de Filippi). *Bull. Soc. Zool. France* **24:** 181–189.

Fernandez, J. 1980. Embryonic development of the glossiphoniid leech *Theromyzon rude*: characterization of developmental stages. *Dev. Biol.* **76:** 245–262.

Fernandez, J., and G. S. Stent. 1980. Embryonic development of the glossiphoniid leech *Theromyzon rude*: structure and development of the germinal bands. *Dev. Biol.* **78:** 407–434.

Filippi, F. de. 1849a. Über eine neue riesengrosse Egelart. *Z. Wiss. Zool.* **1:** 256–257.

Filippi, F. de. 1849b. Sopra un nuovo genre (*Haementeria*) di Annelidi della famiglia della Sanguisughe. *Mem. Acc. Sci. Torino* (2) **10:** 1–14.

Herter, K. 1936. Die Physiologie der Hirudineen. P. 126 in H. G. Bronn, Ed., *Klassen und Ordnungen des Tierreichs* Vol. 4, Div. III, Book 4, Part 2. Akad. Verlagsgesellschaft, Leipzig.

Lang, A. 1890. Über die äussere Morphologie von *Haementeria ghilianii*, F. de Filippi. *Festschrift zum 50. Doktorjubiläum von Nägeli und Kölliker.* Zurich, pp. 199–211.

Leydig, F. 1862. Über das Nervensystem der Anneliden. *Arch. Anat. Physiol. Lpz.* **1862:** 90–124.

Mann, H. K. 1962. *Leeches (Hirudinea).* Pergamon Press, Oxford. 201 pp.

Myers, R. J. 1935. Behavior and morphological changes in the leech, *Placobdella parasitica*, during hypodermic insemination. *J. Morphol.* **57:** 617–648.

Nicholls, J. G., and S. W. Kuffler. 1964. Extracellular space as a pathway for exchange between blood and neurons in the central nervous system of the leech: ionic composition of glial cells and neurons. *J. Neurophysiol.* **27:** 654–671.

Nicholls, J. G., and D. Van Essen. 1974. The nervous system of the leech. *Sci. Amer.* **230:** 38–48.

Retzius, G. 1891. Zur Kenntniss des centralen Nervensystem der Würmer. *Biol. Unters.* (*N.F.*) **2:** 1–28.

Sawyer, R. 1978. Domestication of the world's largest leech for developmental neurobiology. Pp. 212–213 in *Year Book of American Philosophical Society*, Philadelphia, Pa.

Schleip, W. 1936. Ontogenie der Hirudineen. Pp. 1–121 in H. G. Bronn, Ed., *Klassen und Ordnungen des Tierreichs* Vol. 4, Div. III, Book 4, Part 2. Akad. Verlagsgesellschaft, Leipzig.

Stent, G. S., W. B. Kristan, Jr., W. O. Friesen, C. A., Ort, M. Poon, and R. L. Calabrese. 1978. Neuronal generation of the leech swimming movement. *Science* **200:** 1348–1357.

Weisblat, D. A., G. Harper, G. S. Stent, and R. T. Sawyer. 1980. Embryonic cell lineages in the nervous system of the glossiphoniid leech *Helobdella triserialis*. *Dev. Biol.* **76:** 58–78.

Weisblat, D. A., R. T. Sawyer, and G. S. Stent. 1978. Cell lineage analysis by intracellular injection of a tracer. *Science* **202:** 1295–1298.

Whitman, C. O. 1878. The embryology of Clepsine. *Q. J. Microsc. Sci.* **18:** 215–315.

Whitman, C. O. 1887. A contribution to the history of germ layers in Clepsine. *J. Morphol.* **1:** 105–182.

Reference: *Biol. Bull.*, **160:** 332–347. (April, 1981)

FACTORS AFFECTING OXYGEN CONSUMPTION IN THE MARINE PULMONATE *AMPHIBOLA CRENATA* (GMELIN, 1791)

SANDRA E. SHUMWAY[1]

Portobello Marine Laboratory, University of Otago, Dunedin, New Zealand

ABSTRACT

The rate of oxygen consumption by *Amphibola crenata* is unaffected by the salinity of the external medium in the range 0–125% sea water. There is no significant difference between the rates of oxygen consumption in air and water, oxygen consumption varying with the 0.45 power of body weight. During exposure to anaerobic conditions the snails showed an increase in the post-anaerobic:pre-anaerobic respiratory rate, this ratio reaching a maximum after 6 h anoxia over the range 0–125% sea water. Specimens of *Amphibola crenata* exposed to declining oxygen tensions at full salinity show almost no ability to regulate their rate of oxygen consumption. The ratio K_1/K_2, (see text) plotted against the weight specific oxygen consumption yields the following equation: $K_1/K_2 = 527.7\ QO_2^{1.610}$. As salinity decreases, the ratio K_1/K_2 also decreases, indicating an increase of oxygen independence with decreasing salinities. As salinity decreases, the zone of critical pressure decreases, *i.e.* the snails become more oxygen independent; the maximum decrease in the zone of critical pressure coming in animals exposed to 0 and 25% sea water. When exposed to both salinity and anoxic stress, the animals reach their maximum degree of oxygen independence.

INTRODUCTION

Amphibola crenata (Gmelin, 1791) is a pulmonate gastropod and a monotypic species of the family Amphibolidae, endemic to New Zealand. The snail belongs to a group of pulmonates sometimes termed "gehydrophilous" in which, while the gill has been replaced by a lung, the animal has not yet become truly an inhabitant of fresh water (Farnie, 1919). Morton and Miller (1968) have described *Amphibola* as an archaic pulmonate, still having an operculum and veliger larva, and place it at a "lung fish" stage of gastropod evolution.

Amphibola is found in vast numbers on mud flats in sheltered bays, inlets, and estuaries. Wherever it occurs, especially on mud flats, the snail is usually the most noticeable and dominant animal. In spite of this, very little work has been done concerning the animal's ecology and none concerning its physiology. Watters (1964) published a detailed study of the distribution of *Amphibola* in the area used in the present study and recorded snails in salinities ranging from 3–30.5‰ with the majority in salinities of 22–25‰. Personal observations indicate that *Amphibola* occurs in the study area in salinities ranging from 0–40‰.

Amphibola is a detritus feeder. Watters (1964) reported that the snails tend

---

Received 18 July 1979; accepted 5 December 1980.

Abbreviations: VO_2 = rate of oxygen uptake, QO_2 = weight specific oxygen consumption, PO_2 = oxygen tension, K_1/K_2 = ratio indicating oxygen dependence, P_c = zone of critical pressure (for *A. crenata* oxygen cosumption).

[1] Present address: Department of Ecology and Evolution, State University of New York, Stony Brook, L. I., N. Y., 11794.

to burrow under mud or sand as the tide rises and remain there until the tide recedes. Briggs (1972) found that *A. crenata* is submerged for only approximately 1–2 h in any one tidal cycle. Thus, the animals may be exposed to anoxic conditions for up to about 2 h. Over a tidal cycle, the oxygen concentration of the ambient water may drop by as much as 75% (personal observation). In addition, in many cases, the snails are not covered by sea water during each tidal cycle and may remain exposed for several weeks.

While many studies deal with effects of salinity on respiration in marine invertebrates and several with effects of declining oxygen tension, only Bayne (1973) has examined the combined effects of salinity and declining oxygen tension on respiration.

Amphibola occupies a transitional habitat between marine and terrestrial conditions and is one of the very few holoeuryhaline species known. Thus, the present study was undertaken to compare its respiratory capabilities with those of marine and freshwater gastropods.

MATERIALS AND METHODS

Specimens of *Amphibola crenata* (Gmelin, 1971), 0.004–1.0 g dry weight (approximately 0.75–3.5 cm shell height) were collected from Hoopers Inlet, Portobello, New Zealand, and maintained in the laboratory at 15°C for at least 1 week before experiments. Animals were used within three weeks of collection. Shell height and dry tissue weight were determined for 75 freshly collected snails. The linear regression relating shell height to tissue dry weight gave the equation: $Y = 0.022X^{3.01}$ where Y = dry weight of tissue (g), X = shell height (cm), and ($r = 0.961$; $p < 0.001$). After periods of 6 and 12 weeks these same measurements were taken on another 10 animals. There was no evidence of significant weight loss even after 12 weeks in the laboratory.

Aquatic oxygen consumption of *A. crenata* was measured using a Radiometer oxygen electrode according to the method described by Crisp *et al.* (1978). Aerial oxygen consumption was measured using a Gilson differential respirometer. In all cases, individual animals were used for each experiment and experimental chambers ranging from 5–50 ml were used in the aquatic respiration determinations. These chambers were cleaned frequently with hot water and bleach to eliminate bacterial growth. A control (chamber with aerated sea water) was run after every fifth experiment.

For experiments on the effects of salinity on oxygen uptake, Otago Harbour sea water (salinity ≅ 33.5‰) was diluted with rainwater or evaporated to give the appropriate salinity. Animals were placed directly from 100% sea water into the experimental salinity. Therefore, the short-term or acute response to salinity change is reported here. Snails were immediately active upon direct transfer to the salinity range 25–125% sea water. However, animals placed directly into rainwater did not emerge from their shells for the first 2 h. Therefore, animals were placed in 0% sea water for 2 h before the start of the experiments. All experiments were carried out at 15°C.

To determine the effects of declining oxygen tension upon oxygen uptake, the snails were placed in the respirometer chamber and allowed to exhaust the available oxygen. While it may be argued that this method can introduce interference by other metabolites in the respiratory response, it was felt that this method reflected conditions likely to be experienced in the natural environment, where the snails are not likely to experience a sudden decline in available oxygen. In addition, Sassaman and Mangum (1972) simultaneously monitored the oxygen consumption of several species of anemones in closed containers and the pH of the water and found that detectable increases in metabolites were confined to oxygen levels below about 25 mm Hg (approximately 16% air saturation). They concluded that the response of animals exposed to decreasing oxygen tension in sealed containers was due essentially to changes in the oxygen tension. Taylor and Brand (1975a, b) also used the closed chamber technique and found oxygen consumption rates unaffected by metabolite accumulation in bivalves.

For experiments to determine "oxygen debt" or the effect of prolonged exposure to anoxia, animals were allowed to deplete the oxygen supply and then left under anaerobic conditions for different periods, after which the water in the chamber was replaced with well-aerated sea water. Rates of oxygen uptake were again measured or the animals again allowed to deplete the oxygen supply.

For experiments to determine the effects of anoxia and salinity on oxygen uptake in declining oxygen tensions, snails of a narrow size range were used. Variations in the data due to weight differences were minimized by relating all measurements of rate of oxygen uptake (VO_2) to $W^{0.45}$ where W is dry weight (g) and 0.45 is the value of the exponent relating VO_2 to body size ($VO_2 = aW^{0.45}$) (Bayne and Livingston, 1977).

RESULTS

Effect of salinity on oxygen uptake at full oxygen tension

Table I shows the relationship between oxygen consumption in air-saturated sea water and dry body weight, together with the relationship between oxygen consumption in air and dry body weight. The data have been fitted to the equation $Y = aX^b$, where Y = oxygen consumption in ml/h (VO_2) and X = dry weight in g. The equation parameters are given in Table I. While it appears that oxygen uptake rates are higher in water than in air, the difference is not significant.

The effect of salinity on the rate of oxygen uptake (VO_2), and the equation parameters are also given in Table I. There is no significant difference between any of the lines; however, the rate in 125% sea water appears slightly lower than the rate in other salinities. It is possible that with a large sample size these lines could be separated.

Oxygen debt—the effect of hypoxia on oxygen consumption

The effects of anoxia on the rate of oxygen consumption at different experimental salinities are expressed in Table II in terms of a possible oxygen debt, *i.e.*

TABLE I

Linear regression equation of oxygen consumption (log 10^Y) on dry weight ($\log^{X \cdot g}$); n = number of determinations; r = correlation coefficient. See text for details.

Experimental conditions	a	b	n	*r*	*P*
Aerial	0.138	0.46	42	0.56	0.001
125% SW	0.108	0.43	30	0.84	0.001
100% SW	0.158	0.45	70	0.89	0.001
75% SW	0.139	0.44	30	0.88	0.001
50% SW	0.134	0.45	30	0.60	0.001
25% SW	0.128	0.46	35	0.88	0.001
0% SW	0.141	0.43	25	0.86	0.001

TABLE II

Effect of salinity on the "oxygen debt" in Amphibola crenata *exposed to zero oxygen tension. N = 10 for each determination. Oxygen debt as (VO_2 after hypoxia ÷ VO_2 before hypoxia) ± SD*

% Seawater	Hours hypoxia				
	2	4	6	12	24
125	1.04 ± 0.10	1.48 ± 0.02	1.51 ± 0.10	1.61 ± 0.09	1.69 ± 0.13
100	0.93 ± 0.21	1.48 ± 0.06	1.59 ± 0.04	1.64 ± 0.10	1.76 ± 0.05
75	1.01 ± 0.06	1.31 ± 0.11	1.40 ± 0.04	1.61 ± 0.06	1.80 ± 0.10
50	1.24 ± 0.10	1.51 ± 0.15	1.41 ± 0.08	1.81 ± 0.11	1.69 ± 0.06
25	1.61 ± 0.07	1.70 ± 0.11	1.68 ± 0.08	1.76 ± 0.12	1.79 ± 0.08
0	1.58 ± 0.30	1.53 ± 0.07	1.69 ± 0.10	1.76 ± 0.08	1.74 ± 0.05

the ratio of post-anoxia VO_2 to pre-anoxia VO_2. Values notably greater than one indicate a higher level of oxygen consumption when animals were returned to oxygen-saturated sea water than before exposure to anoxic conditions. Effects of salinity were most pronounced in animals exposed to 2 h hypoxia. Animals in 125%, 100% (control), and 75% sea water showed no major oxygen debt after 2 h hypoxia, whereas animals in more dilute salinities (50, 25, and 0% sea water) showed large oxygen debts. Maximum values were attained in all salinities after 12–24 h exposure to hypoxic conditions.

Oxygen dependence and independence

Several studies concern the effects of declining oxygen tension on oxygen uptake in marine invertebrates and the treatment of data derived from these experiments (Tang, 1933; Bayne, 1971a; Mangum and Van Winkle, 1973; Bayne and Livingston, 1977).

Tang (1933) and Bayne (1971a) used the following simple hyperbolic expression to describe the relationship between the rate of oxygen uptake and ambient oxygen tension:

$$QO_2 = PO_2 \div (K_1 + (K_2 \cdot PO_2)) \qquad (1)$$

where: QO_2 = weight specific oxygen consumption (ml/(h/g dry wt), PO_2 = oxygen tension in mm Hg, and K_1 and K_2 are constants calculated from the linear form of equation (1) or, in more general terms: $Y = X \div (K_1 + K_2X)$.

It is evident that if K_2 is large compared with K_1, then Y will not vary much with X. The limiting case is if K_1 is so small that you can ignore it, in which case X/K_2X is a constant equal to Y.

Similarly, increasing K_1 in relation to K_2 causes greater variation of Y as X varies. It should be noted that as long as K_1 is greater than 0, the equation can never describe complete oxygen independence.

Van Winkle and Mangum (1975) overcame this problem by including an intercept parameter in the equation so that the predicted curve need not pass through the point (X, Y) = (0, 0). Rearranging (1) gives:

$$PO_2/QO_2 = K_1 + K_2 \cdot PO_2 \qquad (2)$$

where: K_1 = intercept, and K_2 = slope of the regression equation relating PO_2/QO_2 to PO_2. This provides an empirical means of finding the values of K_1 and K_2.

Bayne (1971) proposed that the ratio K_1/K_2 provides an index of dependence of oxygen consumption on oxygen tension, since as K_1 increases in relation to K_2 the rate of oxygen consumption, VO_2, becomes more directly proportional to oxygen tension, PO_2 (oxygen dependent species). Conversely, as K_2 increases in relation to K_1, oxygen consumption approaches a constant value (oxygen independent species) for different values of PO_2.

Mangum and Van Winkle (1973) tested a number of equations which might be used to describe the relationship between oxygen consumption and oxygen concentration. The found that for goodness of fit, ease of computation, and useful parameters for interspecific comparisons, a second order polynomial gave the best results:

$$Y = B_0 + B_1X + B_2X^2 \qquad (3)$$

where Y = weight specific oxygen consumption ml/(h · g dry wt), $X = PO_2$, B_0 = minimum rate of oxygen uptake found at very low PO_2, B_1 = linear effect of X on Y, and B_2 = deviation from linearity of the effect of X on Y.

The equation uses standardized data, *i.e.* the initial value is expressed as 1.0 and subsequent values as fractions of 1.0. Thus, for a strict oxyregulator (oxygen independent species) B_0 would equal 1, and B_1 and B_2 would equal zero. A strict oxyconformer (oxygen dependent species) would have both B_0 and B_2 equal to 0 and $B_1 > 0$. Mangum and Van Winkle (1973) also suggested the second order coefficient, B_2, may be used as an index of a species' ability to regulate its rate of oxygen uptake in declining oxygen tensions. The more negative the value of B_2 the more oxygen independent the animal. Both of the above methods make use of the "weight specific" oxygen consumption, QO_2, in their application. Bayne and Livingstone (1977), however, established a treatment for results relating VO_2 (oxygen consumption in ml/h) to PO_2. They fitted both standardized and nonstandardized data relating VO_2 and PO_2 to the three equations shown in Table III. In contrast with the method of Mangum and Van Winkle (1973), these equations were not meant to produce parameters of biological relevance but rather to give a means of relating VO_2 to PO_2 in different experiments.

For comparative purposes and so that the data here might be compared with those already available in the literature, all three approaches have been employed in analyzing the data.

Effect of declining oxygen tension on oxygen consumption at full salinity

A total of 20 experiments relating oxygen consumption to oxygen tension were carried out. The results of six typical experiments are shown in Figure 1. In each

TABLE III

Statistical models used to fit data relating VO_2 to PO_2, and the goodness of fit of these models. K_1, K_2, and K_3 are fitted parameters (after Bayne and Livingstone, 1977).

		Sums of squares of deviations	
	Model	Non-standardized data	Standardized data
Hyperbolic	$VO_2 = \frac{PO_2}{(K_1 + K_2 \cdot PO_2)}$	0.0113	0.2933
Polynomial	$VO_2 = K_1 + K_2 \cdot PO_2 + K_3\colon (PO_2)^2$	0.0150	0.2384
Semilogarithmic	$VO_2 = K_1 + K_2 (\log_{10} PO_2)$	0.0171	0.2712

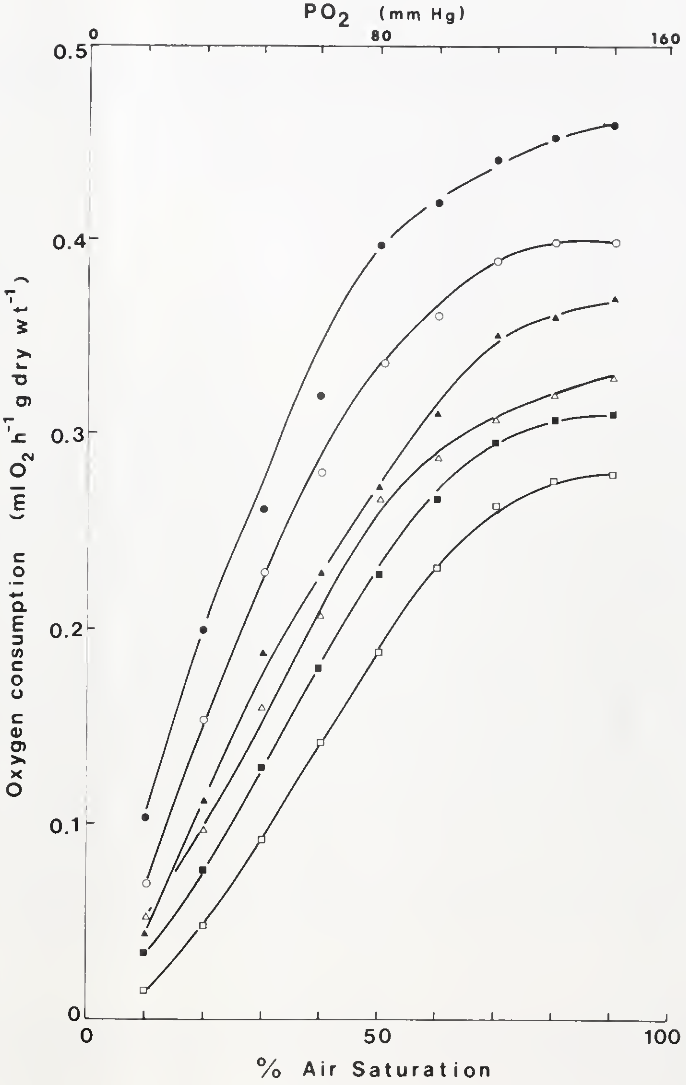


FIGURE 1. Responses of six specimens of *A. crenata* to declining oxygen tension.

experiment the rate of oxygen consumption appeared to be more or less independent of the ambient PO_2 over a narrow range of oxygen tensions.

In order to estimate the degree of this independence, PO_2/QO_2 was plotted against PO_2 (with PO_2 the oxygen tension in mm Hg and QO_2 the weight specific oxygen consumption) (Tang, 1933; Bayne, 1971a, b). As discussed previously, Bayne (1971a, b) proposed the use of the ratio K_1/K_2 as an index of oxygen dependence with smaller values of this ratio, indicating a greater degree of oxygen independence. The values for K_1 and K_2 are given in Table IV.

Figure 2 shows the relationship between the ratio K_1/K_2 and QO_2. The equation of the resultant regression line is: $K_1/K_2 = 527.7QO_2^{1.610}$.

Table IV

Values of K_1 and K_2 in the expression $QO_2 = PO_2/K_1 + K_2\ PO_2$ for seven specimens of A. crenata *of various sizes, subjected to declining oxygen tensions. QO_2, ml O_2/(h·g dry wt); PO_2, mm Hg.*

Animal Number	K_1	K_2	K_1/K_2
1	196	2.14	91.59
2	206	1.60	128.75
3	237	1.45	163.44
4	177	1.72	102.90
5	191	1.27	150.39
6	124	1.54	80.52
7	113	1.30	86.92

Small individuals with high metabolic rates are, therefore, less independent of the ambient oxygen tension than are larger individuals with low metabolic rates.

Effect of time spent in the laboratory on the ability to regulate QO_2

Bayne (1971) showed that the length of time the mussel, *Mytilus edulis*, was maintained in the laboratory could affect the animals' capacity to regulate oxygen uptake in declining oxygen tensions. This possibility was therefore investigated in *A. crenata.*

Animals of similar size were kept in the laboratory for periods of 1, 2, 3, 5, 7, 9, and 12 weeks, after which their response to declining oxygen tension was measured. Table V shows the change in the ratio K_1/K_2 for 70 experiments. It can be seen from these values that, like *Mytilus, A. crenata* loses at least part of its ability to regulate its rate of oxygen consumption in declining oxygen tensions if kept in the laboratory for extended periods.

Combined effects of salinity, declining oxygen tension, and anoxia on oxygen consumption

The effect of salinity on oxygen dependence is shown in Table VI where the oxygen dependence index, K_1/K_2, is given for snails from six different salinities.

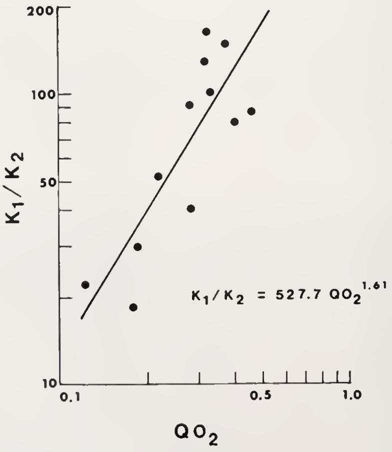


Figure 2. The "oxygen dependence index" K_1/K_2, plotted against weight-specific oxygen consumption.

TABLE V

Oxygen-dependence index for specimens of A. crenata *after different periods in the lab. N = 10 for each experiment.*

Time (weeks)	K_1/K_2
1	93.22
2	90.10
3	98.74
5	312.47
7	415.26
9	510.17
12	905.56

Table VII shows B_2 values (see equation 3) for the same salinities. As the salinity decreases, K_1/K_2 also decreases, indicating an increase of oxygen independence with decreasing salinities. B_2 values become increasingly more negative, also indicating increased oxygen independence.

Figure 3 shows the rates of oxygen consumption during declining oxygen tensions at different salinities after 2 h anoxia. As salinity decreases the zone of critical pressure, P_c, shifts left, *i.e.* the animals become more oxygen independent. In animals placed in 125, 100, and 75% sea water (Line A, Fig. 3) the P_c is between 60–70% air saturation. In animals exposed to O and 25% sea water, the P_c is between 40–50% air saturation. This shift is reflected in the increasingly more negative values of B_2 (Table VII). This gives the animals an increased range of oxygen tensions over which they can maintain an elevated rate of oxygen uptake.

Figure 3 also shows the rates of oxygen consumption during declining oxygen tensions at various salinities in animals previously exposed to 12 h anoxia. Again, this shift if reflected in the B_2 values given in Table VII. In this case the P_c has shifted to the left to a value of 30–40% air saturation, increasing even further the range of oxygen tensions over which they can maintain an elevated rate of oxygen uptake.

DISCUSSION

Little information is available concerning oxygen consumption of marine pulmonates. Berg and Ockleman (1959), studying several species of freshwater pul-

TABLE VI

Oxygen dependence index (K_1/K_2) for specimens of A. crenata *at five different salinities. N = 10; dry weight ≃ 0.3 g.*

Salinity (% SW)	Mean QO_2 in air-saturated SW (ml/(h·g dry wt))	Mean K_1/K_2
125	0.48	90.38
100	0.50	96.24
75	0.43	91.46
50	0.58	66.27
25	0.49	31.14
0	0.52	34.61

TABLE VII

B_2 values ($\times 10^3$) from the expression $Y = B_0 + B_1 X + B_2 X^2$ (see equation 3) for specimens of A. crenata *exposed to declining oxygen tensions and to anoxia at various salinities. $N = 10$ for each experiment.*

Salinity		B_2 ($\times 10^3$)	
% sea water	Normal	After 2 h anoxia	After 12 h anoxia
125	−0.0497	−0.0511	−0.0739
100	−0.0513	−0.0532	−0.0754
75	−0.0502	−0.0567	−0.0740
50	−0.0590	−0.0701	−0.0723
25	−0.0662	−0.0723	−0.0752
0	−0.0681	−0.0721	−0.0749

monates, found the oxygen consumption in relation to weight to vary between less than proportional to surface area and proportional to weight. Brand *et al.* (1948) studied respiration of nine species of freshwater pulmonates with regard to body size but not to the weight exponent b. Their data show an apparent constancy of weight/O_2 relationship within limited size ranges, but also show that the overall picture is distinctly against the validity of such a procedure. For comparative purposes, extrapolating from their graph for oxygen consumption vs. body weight for all pulmonates studied gives an approximate value for b of 0.85. Newell and Pye (1971) report a range of b values for *Littorina littorea* at different temperatures and seasons of 0.245–0.033 for "resting animals." Shumway and Crisp (in preparation) studied oxygen consumption in 30 species of marine gastropods and found values of b ranging from 0.517 to 0.863. The b value of 0.45 reported here for *Amphibola* is relatively low but not unusual and is not affected by salinity changes.

Brand *et al.* (1948) found that the respiratory rate of freshwater pulmonates was higher than that of operculates when snails of equal weight were compared.

In the equation $Y = aW^b$, a is the magnitude of Y (ml/h) when W equals 1 (g dry wt) and can be used to compare oxygen uptake for different animals of the same weight. Shumway and Crisp (in preparation) found "a" values for snails generally similar in size to *Amphibola* to range from 0.128 to 0.166 (*Crepidula fornicata, Calliostoma zizyphinum, Littorina littorea, Patella vulgata*). The value of 0.158 reported here for the marine pulmonate *A. crenata* is within this range and it does not appear that marine pulmonate respiration is any higher than that of marine prosobranchs.

Few authors have studied oxygen consumption of snails in both air and water. Sandison (1966) found that four species of intertidal gastropods all had higher rates of oxygen consumption in air than in water and that in species living at successively higher shore levels the rates of oxygen consumption in air increased. Conversely, McMahon and Russell-Hunter (1977) studied aerial and aquatic oxygen consumption in several species of intertidal prosobranchs and found the aerial uptake rates lower than the aquatic ones in all four intertidal snails studied. Micallef and Bannister (1967) found that aerial and aquatic oxygen consumption in *Monodonta turbinata* were the same at 15°C. They also note that the snail is observed as frequently out of water as immersed. Bannister (1974) measured aerial and aquatic oxygen consumption in *Patella caerulea* (lower eulittoral zone) and *P. lusitanica* (upper eulittoral zone). He found that the rate of oxygen consumption of *P. lusitanica* was about three times higher in air than in water and that the rate of oxygen consumption of *P. caerulea* in water was about twice that in air. Thus,

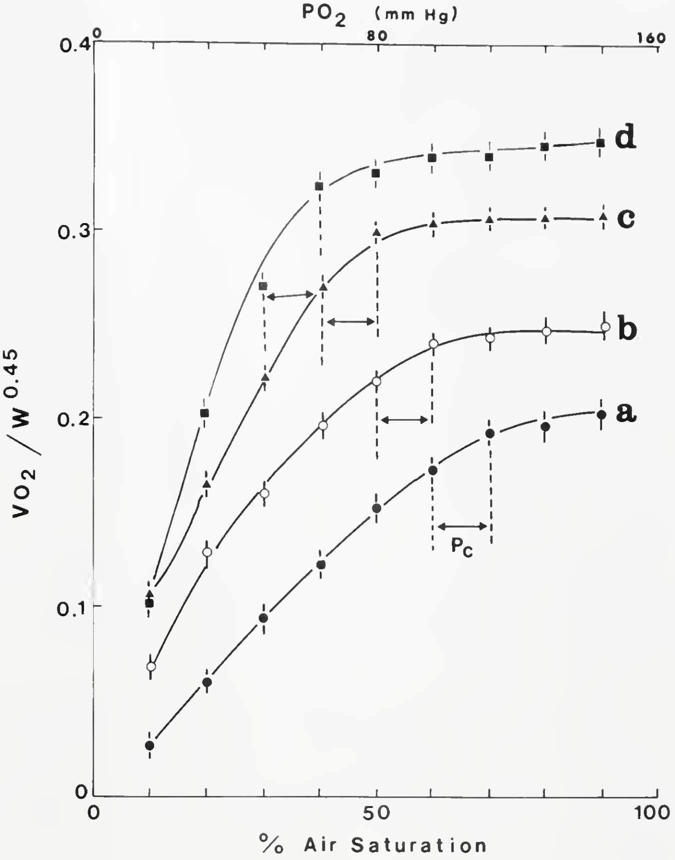


FIGURE 3. The rates of oxygen consumption ($VO_2/W^{0.45}$, ml $O_2^{h^{-1}}$) by *Amphibola crenata* in declining oxygen tensions (PO_2: mm Hg) at different salinities after two and 12 h exposure to anoxia. Solid circles: 125, 100, and 75% sea water and 2 h anoxia; open circles: 50% sea water and 2 h anoxia; triangles: 25 and 0% sea water and 2 h anoxia; solid squares: all salinities and 12 h anoxia. P_c = zone of critical pressure. N = 10 for each salinity tested.

all three studies indicate that respiratory rates in air and water vary according to whether the animals are exposed or submerged.

Accordingly, it might be expected that *Amphibola* would show a higher rate of oxygen consumption in air than in water. This is not the case (Table I). The one major difference between the snails previously studied and *A. crenata* is that *Amphibola* is a pulmonate, and the others are prosobranchs. *Amphibola* employs the mantle cavity as a lung and has been described as being at a "lung-fish" stage of gastropod evolution (Morton and Miller, 1973). As this snail occupies a transitional habitat between marine and terrestrial conditions it is not surprising that it can use aerial and aquatic oxygen equally well. Possibly evolutionary position, not position on the shore, influences the snail's respiratory capabilities.

Kinne (1971) has divided the effects of salinity on the rate of oxygen consumption in marine and brackish-water invertebrates into four categories: (1) increase in subnormal salinities and/or decrease in supranormal salinities, (2) increase in sub- and supranormal salinities (3) decrease in sub- and supranormal salinities, (4) both remain essentially unaffected.

Several species, predominantly crustaceans, maintain constant rates of oxygen consumption when exposed to different salinities (Krogh, 1939 [*Eriocheir sinensis*]; Gilchrist, 1956 [*Artemia salina*]; Frankenberg and Burbanck, 1963 [*Cyathura polita*]; and McLusky, 1968 [*Corophium volutator*]). The results reported here place *Amphibola crenata* in group (4) also. One other snail, *Theodoxus fluviatilis*, has the same oxygen consumption whether it lives in brackish or fresh water (Lumbye, 1958). In his experiments, Lumbye collected animals from either brackish (11.1‰ salinity) or fresh water and measured the animals' oxygen consumption in the same concentration of sea water from which they were collected. Thus, it is not clear whether snails adapted to life in fresh water would show the same rate of oxygen consumption if exposed to brackish water or vice versa. Possibly, although the species is found in both brackish and fresh water, the two populations are preadapted to life at a particular salinity and not to changes in salinity. Lumbye attributes his findings that both populations have the same rate of oxygen consumption to the fact that *T. fluviatilis* immigrated into fresh water in the early interglacial period, thus giving the animal a longer time to adapt to fresh water than other species that have migrated from the sea to fresh water (*e.g. Potamopyrgus jenkinsi,* first recorded from fresh water in Denmark in 1945) (Lumbye, 1958).

Amphibola crenata, unlike *T. fluviatilis*, is not known from areas of exclusively fresh water, although it is found where it is covered with fresh water for considerable periods. During these periods, the animals carry on their normal activities, including mating and deposition of egg masses (personal observation).

The causes of salinity effects on oxygen consumption rates are not clear. Kinne (1971) states that immediate temporary elevation of oxygen consumption following salinity change may result from an increased overall alertness by the animal to counteract physiological stress. If this is the case, it is not surprising that an animal such as *Amphibola*, which occurs naturally in areas which experience large salinity fluctuations, shows no change in its rate of oxygen consumption when exposed to changing salinities.

Many marine invertebrates tolerate anaerobic conditions for varying periods (Theede, 1973). To survive without oxygen, they must use non-oxidative sources of energy, with the accumulation of end products such as lactic acid, alanine, and succinate (see Mehlman and von Brand, 1951; Livingstone and Bayne, 1977; de Zwaan, 1977). When the animals are returned to aerobic conditions, the by-products of anaerobic metabolism must either be excreted or oxidized. Excretion of all end products will not cause an appreciable increase in oxygen consumption; however, oxidation will increase oxygen demand by tissues over normal metabolic requirements, repaying an oxygen debt. Such payment is a short term phenomena and, as will be seen later, the increased post-anoxic respiratory rates recorded here probably are not related to oxygen debt but rather to a compensatory shift in the respiratory response to low oxygen.

The amount of "oxygen debt" incurred has been related to the species' resistance to anoxia—animals with low resistance showing the greatest oxygen debts. Several species of anemones increase their oxygen consumption after exposure to anoxic conditions (Brafield and Chapman, 1965; Sassaman and Mangum, 1972). In addition, Sassaman and Mangum (1972) found that the burrowing anemone *Haloclava producta* has a survival rate under anoxic conditions at least twice that of the epifaunal anemone *Metridium senile* (even though both species survive anoxia for considerable periods). Mehlman and von Brand (1951) studied 11 species of freshwater snails and found that those with considerable resistance to low oxygen concentrations appeared to accumulate no oxygen debt after anoxia, whereas those with a low tolerance to anoxia accumlated lactic acid and repaid a large oxygen debt. McMahon and Russell-Hunter (1978) studied six species of marine snails. In the species most likely to encounter low oxygen concentrations in their natural environments, no oxygen debt was repaid after 5 h anoxia. McMahon (1973) has shown that the freshwater limpet *Laevapex fuscus*, commonly found in reducing substrates, incurred no oxygen debt after 48 h anoxia. Bayne and Livingstone (1977) found a positive oxygen debt in only one experiment, using *Mytilus edulis* that had been exposed to hypoxic conditions for 5 h.

Amphibola kept at 100% sea water showed no post-anoxic increase in respiratory rate after 2 h anoxia but showed a gradual increase in the post-anoxic/pre-anoxic respiratory rate between 4 and 24 h anoxia (increase from 1.48–1.76). The post-anoxic respiratory rate was also influenced by salinity. As the salinity of the medium decreased, the post-anoxic/pre-anoxic respiratory rate increased during exposure to short term anoxia (2 h) but no clear pattern was seen for responses evoked after longer periods (4–24 h).

Amphibola kept at 100% sea water showed no post-anoxia increase in respiratory rate after 2 h anoxia but showed a gradual increase in the post-anoxic/pre-anoxic respiratory rate between 4 and 24 h anoxia (increase from 1.48–1.76) Salinity also influenced the post-anoxic respiratory rate. As the salinity of the medium decreased, the post-anoxic/pre-anoxic respiratory rate increased during short term anoxia (2 h). But there was no clear pattern in responses after longer periods (4–24 h).

While *Amphibola* tolerates low oxygen tensions and anoxic conditions, it probably does not regularly encounter long periods of total anoxia in its natural environment. Briggs (1972) estimated that the snails were only covered for approximately 1–2 hours in any one tidal cycle. We have already seen (Table II) that post-anoxic respiratory rates do not increase appreciably over this period unless the salinity is extremely low—unlikely during a flowing tide. During low tide the snails respire in air. Thus, the snails seem to be well adapted for short periods of anoxia.

The maximum ratio of post-anoxic:pre-anoxic respiration recorded never exceeded approximately 1.8, *i.e.* the rate of oxygen consumption after anoxic exposure was 1.8 times higher than the initial rate. Oxygen consumption probably becomes diffusion-limited beyond this point, and respiration cannot proceed any faster.

The relationship between oxygen consumption and tension has been described by calling animals either oxyregulators (oxygen consumption independent of ambient oxygen concentration) or oxyconformers (oxygen consumption dependent on ambient oxygen concentration). As pointed out by Mangum and Van Winkle (1973) few species exactly fit either category. Molluscs range from conformity to regulation (Von Brand and Mehlman, 1953; Bayne, 1967, 1971a, b; Kushins and Mangum, 1971, McMahon, 1973; Mangum and Van Winkle, 1973; McMahon and Russell-Hunter, 1974; Akerlund, 1974; Taylor and Brand, 1975a, b; Bayne *et al.*, 1976; Bayne and Livingstone, 1977; McMahon and Russell-Hunter, 1978). Bayne (1971a), and Mangum and Van Winkle (1973) put forth means of dealing with these intermediate curves (see results section). The polynomial provides the best fit for standardized data in agreement with the findings of Mangum and Van Winkle (1973) (Table III). The B_2 ($\times 10^3$) value for *A. crenata* was −0.0513, intermediate between the values reported by Mangum and Van Winkle (1973) for two species of gastropods (*Lunatia heros* 0.0489 and *Urosalpinx cineria* −0.1180). McMahon and Russell-Hunter (1978) report values of B_2 for six species of snails ranging from −0.0403 to −0.0868. Table VIII gives the K_1/K_2 values for *Amphibola*

TABLE VIII

The regression equations for the oxygen-dependence index, K_1K_2, against the weight specific oxygen consumption QO_2.

Species	Regression equation	Number of determinations	Source
Geloina ceylonica	$K_1/K_2 = 3.88QO_2^{0.457}$	10	Bayne (1973)
Anadara granosa	$K_1/K_2 = 6.46QO_2^{0.615}$	7	Bayne (1973)
Modiolus demissus	$K_1/K_2 = 62.71QO_2^{0.930}$	10	Shumway (1979)
Mytilus edulis	$K_1/K_2 = 75.50QO_2^{0.818}$	10	Bayne (1971)*
Chlamys delicatula	$K_1/K_2 = 115.78QO_2^{0.769}$	6	McKay & Shumway (1980)
Laevicardium crassum	$K_1/K_2 = 550.00QO_2^{1.829}$	10	Bayne (1971)*
Amphibola crenata	$K_1/K_2 = 527.70QO_2^{1.610}$	12	Present study

* See Mackay and Shumway (1980).

along with values recorded for other species of molluscs. As in other species studied (Bayne, 1971a, b; Taylor and Brand, 1975b; Mackay and Shumway, 1980) it was found that the ratio K_1/K_2 for *A. crenata* declines with decreasing QO_2 and, therefore, with increasing body size, *i.e.* the animals show an increase in respiratory independence with increasing size. This is reflected in the snail's distribution: Small animals (with little or no capabilities for respiratory independence) are found predominantly in well-aerated, high-salinity water.

Both the large K_1/K_2 value and B_2 value indicate that *Amphibola* has little capacity for regulating its rate of oxygen consumption in 100% sea water without prior anoxic stress.

As pointed out by Lange *et al.* (1972), many studies deal with the effects of declining oxygen tension on oxygen consumption, but relatively few discuss the effects of other factors such as salinity and previous exposure to anoxia on the animals' response to declining oxygen tension.

Bayne (1973) studied the responses of three species of bivalve molluscs to declining oxygen tension at reduced salinity. In all three species, capacity to regulate oxygen consumption in declining oxygen tension decreased with reduced salinity, *i.e.* the ratio K_1/K_2 increased with decreasing salinity. This is opposite to the response shown by *A. crenata* (Table VI), where the ratio K_1/K_2 decreased with decreasing salinity, indicating an increased capacity for regulating oxygen consumption with decreased salinity. This difference may not be as startling as it appears. Bayne (1973) also points out that while the stenohaline species, *Anadara*, cannot maintain a high oxygen uptake in declining oxygen tensions at reduced salinities, two more euryhaline species, *Geloina* and *Mytilus*, maintained higher rates of oxygen uptake under the same conditions. *Amphibola* has a "preferred" salinity equal to approximately 60% sea water, (Shumway and Freeman, in prep.) and it is therefore perhaps not surprising that this species shows maximum capacity to regulate oxygen consumption at salinities of 0–15‰ (0–50% SW) and loses the capacity in salinities above approximately 15‰ (50% SW). There also is no change in K_1/K_2 for *Amphibola* in the range 75–125% sea water.

Van Winkle and Mangum (1975) pointed out that temperature or salinity might modify the value of B_2. Table VII indicates that in *A. crenata*, the lower the salinity, the lower the value of B_2. Again, this indicates increased regulation in decreased salinities.

McMahon and Russell-Hunter (1978) studied the respiratory responses to low oxygen stress in six species of snails exposed to varying periods of anoxia. Pre-stress respiratory patterns shifted to a post-stress pattern of greater oxygen independence, particularly at low oxygen tensions. These authors feel that post-stress increases in oxygen uptake rates and greater regulation of oxygen uptake are a short-term compensatory response that allows the snails to maintain relatively high aerobic metabolic rates, instead ot switching to less efficient anaerobic pathways, during occasional low oxygen concentrations. *A. crenata* (see Fig. 3) responded similarly to 12 h anoxia. The zone of critical oxygen pressure shifted from about 60–80% to about 30–40% air saturation. It is presumed that this response, like the one previously reported by McMahon and Russell-Hunter, is short-term, allowing the snails to maintain high rates of oxygen consumption in declining oxygen tensions. (for instance, when repaying an oxygen debt).

An animal such as *Amphibola* may encounter anoxia and salinity stress simultaneously in its natural environment. Salinity did not affect oxygen consumption by *A. crenata* at concentrations near oxygen saturation, but post-anoxic oxygen consumption depended on salinity as well as length of anoxic exposure. After a 2 h anoxia (Fig. 2, 3), salinity has a pronounced effect on the snails' capacity to regulate oxygen consumption in declining oxygen tensions. Over this period, as the salinity decreases, the zone of critical pressure shifts to the left, so that for maximum salinity stress (0–25% sea water) the zone of critical pressure has shifted to about 40–50% air saturation. Since these snails are unlikely to be exposed to anoxia for more than about 2 h at any one time, this shift, too, probably is a short-term response enabling the animals to avoid reverting to anaerobic metabolism.

After 12 h anoxia (*i.e.* when the post-anoxic/pre-anoxic respiration rate is at its maximum) salinity no longer prominently affects the zone of critical pressure and the P_c has shifted even farther to the left, into about 30–40% air saturation, for all salinities tested. This is also reflected in the values for B_2 for varying periods of anoxic stress (Table VII).

In *Amphibola*, the long-term post-anoxic elevations in oxygen consumption apparently are not related to an "oxygen debt" payment but rather to compensatory shifts in the respiratory response to low oxygen, involving increased oxygen consumption at all oxygen tensions and a marked shift toward oxygen independency (see Fig. 3). Such respiratory compensation has been described for *Mytilus edulis* (Bayne, 1973) and for several species of prosobranchs (McMahon and Russell-Hunter, 1978). It should also be noted that longer periods of anoxia increase the rate of oxygen consumption at full oxygen saturation and that long-term anoxia causes greater shifts toward regulation of oxygen consumption. Thus, in *Amphibola crenata*, salinity and anoxia both affect the response to declining oxygen tensions in the short-term, whereas only anoxia affects the respone in the long-term.

ACKNOWLEDGMENTS

I wish to thank Professor R. F. H. Freeman, Dr. J. B. Jillett and an anonymous reviewer for critically reading the manuscript, and Dr. I. D. Marsden for the use of the Gilson respirometer and helpful discussion. Professor G. H. Satchell and Dr. P. A. Sullivan also loaned essential equipment. This work was carried out at the

Portobello Marine Laboratory under tenure of an Otago University Post-doctoral Fellowship, for which I am most grateful.

LITERATURE CITED

AKERLUND, G. 1974. Oxygen consumption in relation to environmental oxygen concentrations in the ampullariid snail *Marisa cornuatietis* (L.) *Comp. Biochem. Physiol.* **47:** 1065–1075.

BANNISTER, W. H. 1974. The respiration in air and water of the limpets *Patella caerulea* (L.) and *Patella lusitanica* (Gmelin). *Comp. Biochem. Physiol.* **49:** 407–411.

BAYNE, B. L. 1967. The respiratory response of *Mytilus perna* L. (Mollusca: Lamellibranchia) to reduced environmental oxygen. *Physiol. Zool.* **40:** 307–313.

BAYNE, B. L. 1971a. Oxygen consumption by three species of lamellibranch molluscs in declining ambient oxygen tension. *Comp. Biochem. Physiol.* **40:** 965–970.

BAYNE, B. L. 1971b. Ventilation, the heart rate and oxygen uptake by *Mutilus edulis* L. in declining oxygen tension. *Comp. Biochem. Physiol.* **40:** 1065–1085.

BAYNE, B. L. 1973. The responses of three species of bivalve mollusc to declining oxygen tension at reduced salinity. *Comp. Biochem. Physiol.* **45:** 793–806.

BAYNE, B. L., C. J. BAYNE, T. C. CAREFOOT AND R. J. THOMSON. 1976. The physiological ecology of *Mytilus californianus* Conrad. 2. Adaptations to low oxygen tension and air exposure. *Oecologia* **22:** 229–250.

BAYNE, B. L. AND D. R. LIVINGSTONE. 1977. Responses of *Mytilus edulis* L. to low oxygen tension: Acclimation of the rate of oxygen consumption. *J. Comp. Physiol.* **114:** 129–142.

BAYNE, B. L., R. J. THOMSON, AND J. WIDDOWS. 1973. Some effects of temperature and food on the rate of oxygen consumption by *Mytilus edulis* L. Pp. 181–193 in W. Weiser, Ed., *Effects of Temperature on Ectothermic Organisms.* Springer Verlag, Berlin.

BERG, K., AND K. W. OCKELMANN. 1959. The respiration of freshwater snails. *J. Exp. Biol.* **36:** 690–708.

BERTALANFFY, L. VON. 1957. Quantitative laws in metabolism and growth. *Q. Rev. Biol.* **32:** 217–231.

BRAFIELD, A. R., AND G. CHAPMAN. 1965. The oxygen consumption of *Pennatula rubra* Ellis and some other anthozoans. *Z. Vergl. Physiol.* **50:** 363–370.

BRAND, T. VON, M. O. NOLAN, AND E. R. MANN. 1948. Observations on the respiration of *Australorbis glabratus* and some other aquatic snails. *Biol. Bull.* **95:** 199–213.

BRIGGS, A. 1972. Feeding studies in *Amphibola crenata.* M. Sc. Thesis. University of Auckland, New Zealand.

CRISP, M., J. DAVENPORT, AND S. E. SHUMWAY. 1978. Effects of feeding and of chemical stimulation on the oxygen uptake of *Nassarius reticulatus* (Gastropoda: Prosobranchia). *J. Mar. Biol. Assoc. U. K.* **58:** 387–399.

DEZWAAN, A. 1977. Anaerobic energy metabolism in bivalve molluscs. *Oceanogr. Mar. Biol. Ann. Rev.* **15:** 103–187.

FARNIE, W. C. 1919. The structure of *Amphibola crenata* (Martyn). *Trans. Proc. N. Z. Inst.* **51:** 69–85.

FRANKENBERG, D., AND W. D. BURBANCK. 1963. A comparison of the physiology and ecology of the estuarine isopod *Cyathura polita* in Massachusetts and Georgia. *Biol. Bull.* **125:** 81–95.

GILCHRIST, B. M. 1956. The oxygen consumption of *Artemia salina* (L.) in different salinities. *Hydrobiologia* **8:** 54–65.

KINNE, O. 1971. Salinity: animals—invertebrates. Pp. 821–995 in O. Kinne, Ed., *Marine Ecology* Vol. 1. Environmental Factors, Part 2. Wiley Interscience, London.

KROGH, A. 1939. *Osmotic Regulation in Aquatic Animals.* Cambridge Univ. Press, London. 242 pp.

KUSHINS, L. J., AND C. P. MANGUM. 1971. Responses to low oxygen conditions in two species of the mud snail *Nassarius. Comp. Biochem. Physiol.* **39:** 421–435.

LANGE, R., H. STAALAND, AND A. MOSTAD. 1972. The effect of salinity and temperature on solubility of oxygen and respiratory rate in oxygen-dependent marine invertebrates. *J. Exp. Mar. Biol. Ecol.* **9:** 217–229.

LIVINGSTONE, D. R., AND B. L. BAYNE. 1977. Responses of *Mytilus edulis* L. to low oxygen tension: Anaerobic metabolism of the posterior adductor muscle and mantle tissues. *J. Comp. Physiol.* **114:** 143–155.

LUMBYE, J. 1958. The oxygen consumption of *Theodoxus fluviatilis* (L.) and *Potamophyrgus jenkinsi* (Smith) in brackish and fresh water. *Hydrobiologia* **10:** 245–262.

MACKAY, J., AND S. E. SHUMWAY. 1980. Factors affecting oxygen consumption in the scallop *Chlamys delicatula* (Hutton) *Ophelia* **19:** 19–26.

MCLUSKY, D. 1968. The oxygen consumption of *Corophium volutator* in relation to salinity. *Comp. Biochem. Physiol.* **29:** 743–753.

MANGUM, C. P., AND W. VAN WINKLE. 1973. Responses of aquatic invertebrates to declining oxygen conditions. *Am. Zool.* **13:** 529–541.

McMAHON, R. F. 1973. Respiratory variation and acclimation in the freshwater limpet, *Laevapex fuscus. Biol. Bull.* **145:** 492–508.

McMAHON, R. F., AND W. D. RUSSELL-HUNTER. 1974. Responses to low oxygen stress in relation to the ecology of littoral and sublittoral snails. *Biol. Bull.* **147:** 490.

McMAHON, R. F., AND W. D. RUSSELL-HUNTER. 1977. Temperature relations of aerial and aquatic respiration in six littoral snails in relation to their vertical zonation. *Biol. Bull.* **15:** 182–198.

McMAHON, R. F. AND W. D. RUSSELL-HUNTER. 1978. Respiratory responses to low oxygen stress in marine littoral and sublittoral snails. *Physiol. Zool.* **51:** 408–424.

MEHLMAN, B., AND T. VONBRAND. 1951. Further studies on the anaerobic metabolism of some fresh water snails. *Biol. Bull.* **100:** 199–205.

MICALLEF, H., AND W. H., BANNISTER. 1967. Aerial and aquatic oxygen consumption of *Monodonta turbinata* (Mollusca: Gastropoda). *J. Zool. Lond.* **151:** 479–482.

MORTON, J., AND M. MILLER. 1973. *The New Zealand Seashore.* 2nd edition. Collins, London. 653 pp.

NEWELL, R. C., AND V. I. PYE. 1971. Variations in the relationship between oxygen consumption, body size and summated tissue metabolism in the winkle *Littorina littorea. J. Mar. Biol. Assoc. U. K.* **51:** 315–338.

SANDISON, E. 1966. The oxygen consumption of some intertidal gastropods in relation to zonation. *J. Zool. Lond.* **149:** 163–173.

SASSAMAN, C., AND C. P. MANGUM. 1972. Adaptations to environmental oxygen levels in infaunal and epifaunal sea anemones. *Biol. Bull.* **143:** 657–678.

TANG, P. S. 1933. On the rate of oxygen consumption by tissues and lower organisms as a function of oxygen tension. *Ann. Rev. Biol.* **8:** 260–274.

TAYLOR, A. C., AND A. R. BRAND. 1975a. Effects of hypoxia and body size on the oxygen consumption of the bivalve *Arctica islandica* (L.) *J. Exp. Mar. Biol. Ecol.* **19:** 187–196.

TAYLOR, A. C. AND A. R. BRAND. 1975b. A comparative study of the respiratory responses of the bivalves *Arctica islandica* and *Mytilus edulis* to declining oxygen tension. *Proc. R. Soc. Lond. B. Biol. Sci.* **190:** 443–456.

THEEDE, H. 1973. Comparative studies on the influence of oxygen deficiency and hydrogen sulfide on marine bottom invertebrates. *Neth. J. Sea Res.* **7:** 244–252.

VAN WINKLE, W., AND C. P. MANGUM. 1975. Oxyconformers and oxyregulators: A quantitative index. *J. Exp. Mar. Biol. Ecol.* **17:** 103–110.

VON BRAND, T., AND B. MEHLMAN. 1953. Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some freshwater snails. *Biol. Bull.* **104:** 301–312.

WATTERS, P. A. 1964. Distribution of *Amphibola crenata* (Pulmonata) in the Dunedin area, with notes on the probable origin of the species. *Trans. R. Soc. N. Z.* **4:** 117–134.

WIDDOWS, J. 1978. Combined effects of body size, food concentration and season on the physiology of *Mytilus edulis* L. *J. Mar. Biol. Assoc. U. K.* **58:** 109–124.

ZEUTHEN, E. 1947. Body size and metabolic rate in the animal kingdom. *C. R. Lab. Carlsb. Ser. Chim.* **26:** 15–161.

ZEUTHEN, E. 1953. Oxygen uptake as related to body size in organisms. *Q. Rev. Biol.* **28:** 1–12.

Continued from Cover Two

high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

4. ***A condensed title*** or running head of no more than 35 letters and spaces should appear at the top of the title page.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text sould be headed LITERATURE CITED, and must be typed double spaced on separate pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin.* Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; the most recent issue). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** *The Biological Bulletin* has no page charges. However, authors will be requested to help pay printing charges of manuscripts that are unusually costly due to length or numbers of tables, figures, or formulae. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates or foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than correction of printers' or editors' errors).

CONTENTS

Invited paper:

JACKLET, JON W.
Circadian timing by endogenous oscillators in the nervous system: toward cellular mechanisms 199

ECKBERG, WILLIAM R.
An ultrastructural analysis of cytoplasmic localization in *Chaetopterus pergamentaceus* 228

FÄNGE, RAGNAR, AND ARTUR MATTISSON
The lymphomyeloid (hemopoietic) system of the Atlantic nurse shark, *Ginglymostoma cirratum* 240

GRIFFITH, ROBERT W.
Composition of the blood serum of deep-sea fishes 250

HERNANDORENA, A.
Metabolic significance of the dietary CMP requirement in relation to *Artemia* morphogenesis 265

HILL, BURKE
Respiratory adaptations of three species of *Upogebia* (Thalassinidea, Crustacea) with special reference to low tide periods 272

JOHNSTON, I. S., P. L. JOKIEL, C. H. BIGGER, AND W. H. HILDEMANN
The influence of temperature on the kinetics of allograft reactions in a tropical sponge and a reef coral 280

LASKER, HOWARD R.
Phenotypic variation in the coral *Montastrea cavernosa* and its effects on colony energetics 292

MARTIN, VICKI JOAN, AND MARY BETH THOMAS
The origin of the nervous system in *Pennaria tiarella*, as revealed by treatment with colchicine 303

SAIGUSA, MASAYUKI
Adaptive significance of a semilunar rhythm in the terrestrial crab *Sesarma* 311

SAWYER, ROY T., FRANCOIS LEPONT, DUNCAN K. STUART, AND ANDREW P. KRAMER
Growth and reproduction of the giant glossiphoniid leech *Haementeria ghilianii* 322

SHUMWAY, SANDRA E.
Factors affecting oxygen consumption in the marine pulmonate *Amphibola crenata* (Gmelin, 1791) 332

Volume 160
Number 3

Marine Biological Laboratory
LIBRARY
JUL 21 1981
Woods Hole, Mass.

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DANIEL L. ALKON, National Institutes of Health and Marine Biological Laboratory
FREDERICK B. BANG, Johns Hopkins University
EDWARD M. BERGER, Dartmouth College
STEPHEN C. BROWN, State University of New York at Albany
HARLYN O. HALVORSON, Brandeis University
J. B. JENNINGS, University of Leeds
FRANK J. LONGO, University of Iowa
GEORGE O. MACKIE, University of Victoria
JOEL L. ROSENBAUM, Yale University
HOWARD A. SCHNEIDERMAN, Monsanto Company
DOROTHY M. SKINNER, Oak Ridge National Laboratory
J. RICHARD WHITTAKER, Boston University Marine Program and Marine Biological Laboratory
E. O. WILSON, Harvard University
GEORGE M. WOODWELL, Ecosystems Center, Marine Biological Laboratory

Editor: CHARLES B. METZ, University of Miami

JUNE, 1981

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Single numbers, $10.00. Subscription per volume (three issues), $27.00 (this is $54.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Charles B. Metz, Editor; or Susan Schwartz, assistant editor, at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between June 1 and September 1, and at the Institute For Molecular and Cellular Evolution, University of Miami, 521 Anastasia, Coral Gables, Florida 33134 during the remainder of the year.

Copyright © 1981, by the Marine Biological Laboratory

Second-class postage paid at Woods Hole, Mass., and additional mailing offices.
ISSN 0006-3185

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978). Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the

Continued on Cover Three

Reference: *Biol. Bull.* **160**: 349–365. (June, 1981)

HIGH SALINITY ACCLIMATION BY THE PRAWN *MACROBRACHIUM ROSENBERGII*: UPTAKE OF EXOGENOUS AMMONIA AND CHANGES IN ENDOGENOUS NITROGEN COMPOUNDS

D. A. ARMSTRONG[1,3], K. STRANGE[2], J. CROWE[2,4], A. KNIGHT[1], AND M. SIMMONS[1]

Department of Land, Air and Water Resources[1] and Department of Zoology[2], University of California, Davis, California 95616; College of Fisheries[3], University of Washington, Seattle, WA 98195; and Bodega Marine Laboratory[4], Bodega Bay, California 94923*

ABSTRACT

The freshwater prawn *Macrobrachium rosenbergii* was subjected to a hyperosmotic transfer from 0‰ to 24‰ salinity. Changes in ammonia excretion, blood Na^+, pH, protein, free amino acids (FAA), and ammonia were monitored for 48 h. Following a rapid reduction in ammonia excretion after transfer, ammonia concentrations in exposure water declined for 24 h. These losses could not be attributed to bacterial growth or aerial diffusion. Also during the first 24 h, blood Na^+ increased from 150 to 280 m*M*, still far below ambient Na^+ concentrations of 366 m*M*. Blood ammonia decreased nearly five-fold and protein concentrations in blood declined about 40 g/l. These data may indicate a reversal of normal Na^+/NH_4^+ exchange following a hyperosmotic shock, such that blood Na^+ is hyporegulated using exogenous NH_4^+ as a counter-ion. During the first 24 h after transfer, net ammonia acquired by uptake could be used to increase intracellular ammonia concentrations as a prelude to increased synthesis of FAA. This model may compliment recent evidence of a CL^-/HCO_3^- reversal in fish hyporegulating blood Cl^- in seawater.

INTRODUCTION

The mechanisms of osmoregulation in eurhyhaline invertebrates have been studied and reviewed extensively (Schoffeniels, 1976; Kirschner, 1979; Gilles, 1979). Extracellular osmolarity depends primarily on excretory and extrarenal mechanisms of ion and water regulation (Prosser, 1973), whereas intracellular osmolarity depends on changes in the concentration of intracellular free amino acids (FAA) and other low molecular weight nitrogenous solutes (Gilles, 1975; Bowlus and Somero, 1979).

Like many euryhaline organisms, crustaceans exposed to low salinity environments maintain blood osmolarity and ionic concentrations hyperosmotic and hypertonic to the external medium (Kerley and Pritchard, 1967; Siebers *et al.*, 1972; Mangum *et al.*, 1976). Upon transfer to high salinity environments, blood ionic and osmotic concentrations increase due to efflux of water and inward movement of ions. Cell volume during hypersaline stress is regulated by an increase in intracellular FAA. Concomitant with this is a decrease in ammonia excretion, thought to indicate greater use of endogenous ammonia to synthesize amino acids (Mangum and Towle, 1977; Gilles, 1979).

Received 28 December 1980; accepted 1 April 1981.

Abbreviations: FAA, free amino acids; FW, freshwater; EC, extracellular, IC, intracellular.

* Send reprint requests to the senior author at this address.

The research discussed here concerns the osmoregulatory response of the freshwater prawn *Macrobrachium rosenbergii* subjected to a hyperosmotic shock. *M. rosenbergii* was chosen for this study because of its euryhaline distribution in nature (Johnson, 1967; George, 1969); its tolerance of large salinity changes, as evidenced by strong osmoregulation (Sandifer *et al.*, 1975; Armstrong, unpublished data); and its wide commercial use, for which physiological information may be helpful in culture programs. Of interest were changes in blood and tissue nitrogenous compounds and blood inorganic ions; in particular, changes in patterns of ammonia excretion after freshwater-acclimated animals were transferred to saltwater. The results reported here indicate that during the early phase of acclimation to a hyperosmotic shock, *M. rosenbergii* apparently takes ammonia from the surrounding medium.

The functions of ammonia in osmoregulatory processes have been studied mainly in two respects: first as a constituent of FAA for intracellular osmotic regulation (Siebers *et al.*, 1972; Bishop, 1976); and second as a counter-ion for regulation of blood Na^+ (Evans, 1975; Maetz *et al.*, 1976; Mangum and Towle, 1977; Kirschner, 1979). Previous studies of these two processes have considered the role of ammonia only in terms of endogenous pools. Apart from its toxicity in solution (Campbell, 1973; Colt and Tchobanoglous, 1976; Armstrong *et al.*, 1978) ammonia is linked to osmoregulation only as an internal molecule that an animal somehow must void. Based on results of this study, we propose two complimentary hypotheses that examine the possible uptake of exogenous ammonia for osmoregulatory functions during and following high salinity acclimation.

MATERIALS AND METHODS

Animals

M. rosenbergii juveniles were reared from larvae of second and third generation University of California, Davis, brood stock. About 60 additional animals were obtained from the hatchery of R. Yates, Winters, California. Broods were mixed and mass reared in 400-l freshwater (FW) tanks equipped with biological filtration. Animals were held in this FW system at least 2.5 months before experiments. Their diet consisted of Purina Ration #20, frozen adult *Artemia*, and fish. Animals' weights and lengths differed somewhat within groups and between the groups of different experiments. Average dry weight of more than 220 experimental animals was 0.436 ± 0.081 (standard deviation) g, with a range from 0.131 to 1.120 g. Carapace length (orbit of eye to posterior of carapace) averaged 12.6 ± 0.7 mm and ranged from 8.5 to 18.0 mm. Temperature and pH in rearing tanks averaged 25°C and 8.10. Terminology for ammonia speciation used throughout this report is that of Colt and Tchobanoglous (1976) and Armstrong *et al.* (1978): "ammonia" refers to the sum of $NH_3 + NH_4^+$; NH_3 signifies un-ionized ammonia; and NH_4^+ the protonated, ionized form.

Experimental protocol

Approximately 1 week before excretion experiments, we removed animals from large rearing tanks, placed 15 in each experimental freshwater 40-l aquarium at 27°C, and subsequently fed the animals twice daily. Ambient ammonia was consistently low during this time (0.0 to 3.0 μM) and total nitrite was $<0.43\ \mu M$ (well below concentrations found stressful to larvae of this species; Armstrong *et al.* 1976). From 24 h before experiments began and for their duration, animals were not fed. Time without food therefore ranged from 48 to 72 h depending on the length of tests.

Two salinities were tested for their effect on excretion: 0‰ (FW), and 24‰ made with tap water and Instant Ocean salts. Water stood in bulk overnight before tests; the pH of both salinities averaged 8.28 ± .07 (not significantly different) and was very stable throughout experiments. On the morning of a test, NH_4Cl was added to both salinities to a total ammonia concentration of 30 μM (=510 μg/l). These solutions were then measured into test beakers. This concentration is higher than levels in many FW lakes, streams, or near-shore and pelagic oceanic areas (Hutchinson, 1957; Lee and Boda, 1977), but it is not atypical of values in eutrophic estuaries and their lowland rivers. For instance, ammonia concentrations in the Sacramento-San Joaquin Delta of California range from 2.6 to 26.5 μM NH_4-N, with values up to 70 μM at some stations (State of California, 1976). *Macrobrachium* is endogenous to fresh and brackish water habitats of the Indo-Pacific (Johnson, 1967; George, 1969), feeding in the flooded, organically rich rice plains of India and Bangladesh. An ammonia concentration of 30 μM is probably representative of that in the prawns' natural habitat.

To measure ammonia excretion, shrimp were thoroughly rinsed in de-ionized water and placed one per 250 or 400 ml beaker (containing 200 or 300 ml of water, respectively). Generally, 0.420 g dry wt was the maximum size of animals put in 250 ml containers. Half the beakers contained FW, half 24 ‰ seawater. There was no prior acclimation of animals to the high salinity. This salinity shock is not lethal (Armstrong, unpublished observations). Each beaker was covered with a plastic petri dish penetrated by a glass pipette from an air manifold. Aeration was gentle and constant throughout all tests. Partial immersion in a bath maintained water temperature at 27°C.

A 2.5 ml water sample, taken from each beaker within 1 min after animals were introduced, was considered time zero (T_0). Subsequently, water from each beaker was generally sampled at 1, 2, 4, 8, 12, 16, and 24 h, and at various times up to 48 h during longer tests. For 48 h experiments water was changed completely at 15 and 33 h, with appropriate T_0 samples taken after each change. In this way, ammonia excretion of 40 animals per experiment was monitored through as many as seven time intervals in 24 h. Ammonia in water samples was immediately reacted with phenol-hypochlorite (Solorzano, 1969) and colorimetrically quantified with a Perkin-Elmer 550 spectrophotometer. Newly molted or soft shrimp were not used in experiments, and the data from animals that molted during tests were not included in the analyses presented here.

In a series of experiments designed to measure the ion regulatory ability of *M. rosenbergii*, groups of animals at 27°C were acclimated to salinities between 0‰ and 28‰. Salinity was increased 4‰ per day and each group was kept at its final salinity 48 h before blood was sampled. Time course changes in blood Na^+ and NH_4^+ after a hyperosmotic shock were studied by abruptly transferring groups of animals from 0‰ to 24‰. Blood samples were collected from groups of 8 to 15 shrimp in as many as eight time periods over 48 h; blood was never taken more than once from any animal.

Analytical procedures

Blood samples: About 10 μl of hemolymph was drawn directly from the pericardial sinus of animals sampled as different experimental groups. This sample was transferred to 2.5 ml glass-distilled water and ammonia determined as previously outlined. Sodium was measured in blood transferred to 3 ml of double glass-distilled water containing 1% HNO_3, 0.2% La, and 500 ppm Cs; protein was precipitated and removed by centrifugation. Ion concentrations were determined with a Varian Techtron 1200 atomic absorption spectrophotometer. Blood pH was determined *in vivo* by inserting a combination microelectrode (Microelectrode, Inc., MI-410) directly into the pericardial sinus, taking care not to puncture the hepatopancreas or stomach. Protein concentration and free amino acids were measured from the pellet and supernatant, respectively, after 24 h extraction and 20 min centrifugation (10,000 *g*) of blood transferred to 85% ethanol at 0°C. Protein was redissolved in 0.5N NaOH and assayed by the method of Lowry *et al.* (1951). Primary amines were quantified on a Turner Model 111 fluorometer after reaction with fluorescamine (North, 1975), using a glycine standard. Total osmolarity was measured on a Wescor 5100 B osmometer, calibrated with NaCl, by transferring blood directly to the machine without clotting or freezing; it was assumed that proteins contributed an insignificant portion of the osmotic pressure.

Tissue: Time course changes in tissue FAA were measured by sampling tail muscle from groups of 20 shrimp killed at various times during 48-h excretion experiments. Muscle portions were blotted, dried 48 h at 50°C, weighed, and stored no longer than 2 weeks in stoppered Nalgene vials until analyzed. Dried tissue was pulverized and extracted in 85% ethanol with intermittent shaking for 24 h at 0°C. Samples were then centrifuged at 10,000 *g* for 20 min in the cold, and primary amines were assayed from the supernatant as previously described. Additionally, secondary amines were measured by reaction with isatin in the dark (Boktor, 1971) and were read immediately at 595 nm against a proline standard.

Calculations: Proportions of NH_3 were calculated as described by Armstrong *et al.* (1978); the pK′a used for 24‰ was 9.33 (see Colt and Tchobanaglous, 1976; Armstrong *et al.*, 1978 for reviews of pK′a calculations) and for FW was 9.15.

Data for ammonia, FAA, and proteins were obtained on a dry weight basis or per volume of blood. To convert data to other units the following relationships were used: Total body water of shrimp in FW and 24‰ seawater was 71.9% ± 0.6 (2.56 $\text{g water} \cdot \text{g dry wt}^{-1}$) or 72.1% ± 0.8 (2.61 $\text{g water} \cdot \text{dry wt}^{-1}$) respectively (no significant difference; Armstrong, unpublished data). A representative value of 72% (2.57 $\text{g water} \cdot \text{dry wt}^{-1}$) was used. Of the total body water, 22% was assumed to be extracellular (EC), based on several studies that measured this parameter in crustaceans (Kerley and Pritchard, 1967; Binns and Peterson, 1969; Lang and Gainer, 1969; Spaargaren, 1972), leaving 78% as intracellular (IC) water. Further, we assumed these values were constant over time after the high salinity transfer, although this is an oversimplification required by lack of specific data.

Results of excretion experiments were compared by analysis of covariance to determine if there was a negative weight-specific relationship, as reported in other studies (Bartberger and Pierce, 1976; Nelson *et al.*, 1977). Analysis of variance was used to test the significant of other relationships, such as change in blood pH or ammonia concentration over time as a function of salinity transfer.

Controls

Ammonia could be lost from the water during excretion experiments by three routes: (1) uptake by the shrimp, (2) uptake by bacterial populations increasing over time, and (3) aerial diffusion to the atmosphere. Tests to gauge loss by the latter two routes were as follows: Aerial loss of ammonia was measured by adding autoclaved FW or 24‰ water (pH 8.25, no significant change during tests) plus

30 or 140 μM ammonia to 10 beakers each (total of 40), constantly aerated as previously described. Water samples were taken at T_0 and every 12 h thereafter to 48 h. The rate of loss as μmoles ammonia $\cdot h^{-1}$ was calculated by a least squares regression of ammonia concentration vs. time.

Growth of bacteria during tests and their possible contribution to changes in ambient ammonia concentrations were gauged by direct plate counts from the medium through time, and use of antibiotics. Excretion experiments were performed as described, by placing shrimp individually in FW or 24‰ beakers (200 ml volumes) to half of which 30 mg chloramphenical/l and 20 mg streptomycin/l had been previously added (no prior acclimation of animals). Ammonia was added to beakers for initial concentrations of 6.1 and 140 μM. A series of water samples were taken to measure ammonia excretion and subsamples were pooled for bacterial plating. A standard dilution series of pooled water was plated onto autoclaved peptone beefheart extract in agar, made either with FW or 24‰ (Instant Ocean salts). Colonies were counted after 36 h incubation at 27°C. The number of bacteria/ml calculated, times the volume of water in beakers gave the total number of bacterial cells in the water column through time. These values was multiplied by the factor 2×10^9 cells/mg dry wt (Stanier *et al.*, 1970) to obtain dry bacterial biomass in micrograms. Finally, an average dry weight nitrogen content of 15% (Stanier *et al.* 1970) was used as a multiplier to get micrograms and micromoles of bacterial nitrogen. Assuming that all bacterial growth during experiments was based on autotorphic use of ammonia nitrogen (ignoring heterotrophic use of organics), then changes in bacterial nitrogen over time may reflect the quantitiy of ammonia lost via bacterial uptake.

RESULTS

All control shrimp in FW beakers survived throughout all tests, including eight animals that molted during this time. Animals abruptly transferred to 24‰ also survived, and showed no gross signs of stress. In nearly all cases there was no loss of equilibrium, and animals at both salinities appeared equally active. Several shrimp in 48-h tests developed slight opacity in tail muscle (see Sindermann, 1977, for discussion of stress related to this condition), but none died, even though 10 animals molted during tests (an event likely to amplify any stress due to salinity shock).

Rates of ammonia excretion differed significantly in FW and 24‰ groups ($P < 0.01$, two-way ANOVA) despite sometimes high variability of rates from one time interval to the next, even within a single experimental group. Since analyses of covariance indicated an equivocal relationship between weight-specific ammonia excretion and total dry weight (significant in some time intervals but not in others), rates here are without covariate corrections.

Average rates of excretion of animals in FW were always positive from one time interval to the next, although their magnitude varied considerably during the first 8 h of tests (Fig. 1). Between 0–1 h, about 5.0 μmoles ammonia $\cdot$ g dry $wt^{-1} \cdot h^{-1}$ were excreted, a rate that had declined to 0.2 μmoles $\cdot g^{-1} \cdot h^{-1}$ by the second hour. The consistently very high excretory rate in the first 1 or 2 h probably resulted from stress from netting and washing when animals were placed in beakers. After 8 h in a beaker, excretion stabilized between 0.95 and 1.40 μmoles $\cdot g^{-1} \cdot h^{-1}$ (Fig. 1).

Transfer to high salinity markedly changed ammonia production. Excretion in the first hour was about 0.75 μmoles $\cdot g^{-1} \cdot h^{-1}$, or about 15% of the rate of ammonia

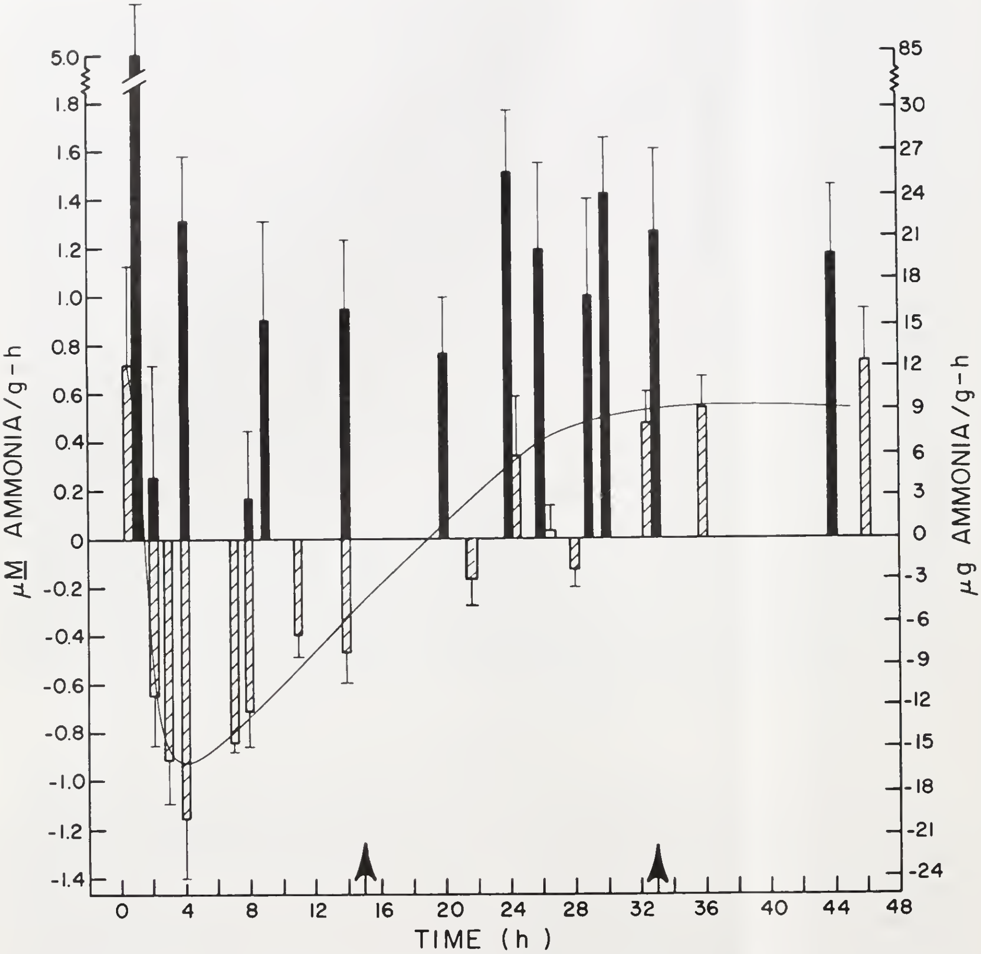


FIGURE 1. Rate of ammonia excretion or uptake by *M. rosenbergii* in FW (solid bars) or transferred to 24 ‰ (hatched bars). Bar height is the mean ± SE of 20–80 animals per time period, based on dry weight. Each rate was calculated in the time interval between it and the preceding bar; for instance, the FW rate at 24 h is the interval between 20–24 h. The solid line represents the trend in ammonia excretion and uptake in 24‰. Arrows indicate water changes.

production by the FW group for this period. About 1 h after the transfer, excretory rates because negative (Fig. 1), as concentrations of ammonia in the medium progressively declined. This apparent uptake reached 1.6 μmoles·g^{-1}·h^{-1} in the 2–4 h interval, approximating the average excretion rate of FW animals between 4 and 48 h. Uptake continued for about 20 to 24 h, gradually declining from 1.16 to 0.7 and 0.5 μmoles·g^{-1}·h^{-1}, at 4, 8, and 14 h, respectively (Fig. 1). Ammonia concentrations in beakers began to increase 24 h after the hyper-saline transfer, indicating animals had resumed net excretion of ammonia, although at a rate of 0.52 μmoles·g^{-1}·h^{-1}, less than half that of FW controls (Fig. 1). Absolute quantities of ammonia lost from beakers during uptake phases, calculated and normalized to body weight, in the 24 h following a hypersaline transfer averaged 12.5 μmoles ammonia·g dry wt^{-1}·h^{-1}.

The ammonia uptake could have represented net losses to the atmosphere and bacteria during periods of very low, yet postive excretion by the shrimp in 24‰ salinity. Controls, however, showed this was not the case. Water of pH 8.25 lost ammonia at a rate of -0.012 ± 0.024 μmoles$\cdot$h^{-1} over 48 h (no shrimp, aerial diffusion control). Multiplying rates at 2, 4, and 8 h (Fig. 1) by 0.436 g dry wt (average weight), the resultant values are -0.283, -0.506, and -0.305 μmoles$\cdot$h^{-1} lost from beakers, some 23 to 42 times greater than the rate of loss to air without animals.

Nor could changes in bacterial biomass as a reflection of autotrophic assimilation of ammonia account for ammonia's disappearance over time (Table I). In water with ambient ammonia levels of 6.1 and 140 μM, increases in bacteria over 24 h represented a possible loss of 0.019 and 0.34 μmoles ammonia, respectively, far less than the net decline of about 12.5 μmoles$\cdot$g^{-1} shrimp calculated above. Also, the rates of greatest ammonia uptake occurred in the first 8 h after transfer, when the slopes of exponential bacterial increase were probably more gradual than in the latter part of the 24-h period, *i.e.*, proportionally less ambient ammonia would be used for bacterial growth early in the incubation than later.

Chloramphenical and streptomycin completely eliminated bacteria from the water column by 4–9 h but had no significant effect ($P > 0.05$, ANOVA) on ammonia uptake between treated and untreated groups in 24‰ (Table II). The magnitude of uptake differed in two experiments at 6.1 and 140 μM ammonia, but there were no significant differences related to antibiotic treatment within an experiment (Table II). However, when experiments were done in FW with initial ammonia concentration of 6.1 μM, antibiotics significantly lowered ammonia excretory rates ($P < 0.01$, ANOVA) in all time intervals studied. For instance, between 4–8 h excretory rates were 0.85 μmoles$\cdot$g$^{-1}\cdot$h^{-1} ($\pm$.23 = SE) with antibiotics and 1.55 ($\pm$.45) without; and between 8–18 h were 0.63 ($\pm$.29) and 2.55 ($\pm$.58)

TABLE I

Increase in bacterial biomass in the water column through time during shrimp excretion experiments, and calculated ambient ammonia required to account for bacterial increases. Values represent eight pooled water samples at 24‰ salinity. Cell count:dry weight ratio was taken as 2 × 10^9 cells/mg dry wt, nitrogen content as 15% of dry weight. See text for details.

Elapsed Time (h)	Initial ammonia (μM)	No. bacteria/ml	No. bacteria/beaker	Bacteria dry wt. (μg)	Calculated ammonia use	
					μg	μmoles $\times 10^{-3}$
0	6.1 (110 μg/l)	—	—	—		—
4	"	1,400	2.03×10^5	0.10	0.015	0.9
9	"	6,500	9.23×10^5	0.46	0.069	4.1
24	"	32,000	4.38×10^6	2.19	0.328	19.0
0	140 (2500 μg/l)	—	—	—		—
5	"	51,000	7.24×10^6	3.62	0.543	32.0
24	"	569,000	7.68×10^7	38.4	5.760	340.0

Table II

Effects of antibiotics (30 mg chloramphenicol/l plus 20 mg streptomycin/l) on rates of apparent ammonia uptake by M. rosenbergii *after transfer from FW to 24‰, and at different initial ambient ammonia concentrations. Rates are given for a common intermediate period (4–8 h), and as averages for entire tests. Negative signs indicate loss of ammonia from beakers (uptake), a positive value shows an increase (excretion).*

Initial ammonia (μM)	+/− Antibiotics	Number animals	Average uptake rates (±SE) (μmoles ammonia$\cdot$g dry wt$^{-1}\cdot$h^{-1})		
			4–8 h	0–18 h	0–36 h
6.1	−	8	−.37 (.11)	.12 (.11)	
6.1	+	8	−.25 (.09)	−.14 (.05)	
140	−	16	−1.36 (.21)		−.41 (.19)
140	+	16	−1.13 (.12)		−.66 (.12)

respectively. If bacteria were using ammonia for growth, then apparent ammonia excretion should have increased or uptake decreased when they were eliminated. Yet either the reverse was true or there was no effect on excretion/uptake rates when bacteria were suppressed.

Concentrations of blood ammonia in the high-salinity group declined significantly ($P < 0.01$, ANOVA, $F = 102.7$, d.f. = 7, 113) by 4 h after transfer to 24‰. It continued to drop for 20 h, from an initial high of 4.82 mM to about 1.0 mM, eventually reaching 0.71 mM by 48 h (Fig. 2). During the same 48 h, blood ammonia of the FW group declined significantly, from 4.82 to 2.94 mM (Fig. 2); still, however, four times higher than in the high salinity group. Blood pH in both treatments increased significantly, although more so in the high salinity, where it rose from 7.58 to 7.87. Blood NH_3 ranged from 23 to 84 μM, while water NH_3 never exceeded 3 μM, indicating a large diffusional gradient from blood to water. NH_3 was 1.8–3.5% of the total blood ammonia and 7.3% of that in water, the balance being ammonium ion (NH_4^+), which was 41–148 times more concentrated in the blood than in water during 24 h incubation (Fig. 2).

Changes in blood concentrations of other nitrogenous compounds were also studied. Protein levels dropped precipitously within 3 h after a transfer to 24‰ salinity, from 107 g protein$\cdot$l^{-1} blood to 63 g$\cdot$l^{-1} (Fig. 3), a decline of 41% ($P < 0.01$, ANOVA, $F = 22.5$, d.f. = 4,35). Following this initial decline, concentrations in blood of animals at 24‰ remained constant at about 65 g$\cdot$l^{-1} through 27 h, while protein levels in blood of FW control shrimp increased slightly but insignificantly to 119 g$\cdot$l^{-1}. Blood FAA changed only slightly over 10 h after transfer to high salinity ($P > 0.05$, ANOVA, $F = 2.04$, d.f. = 3,40) and did not parallel the declines in blood ammonia and protein. Total primary FAA increased from 3.45 to 4.25 m$M\cdot$l^{-1} blood and declined back to 3.5 m$M\cdot$l^{-1} by 10 h after transfer to 24‰ (Fig. 4).

Concentrations of both primary and secondary amines in tail muscle increased significantly 24 h after transfer to 24‰ ($P < 0.01$, ANOVA, $F = 14.00$, d.f. = 4,88) but appeared not to have reached steady states by 48 h (Fig. 5). Total primary amines increased from 570 to 678 m$M\cdot$kg dry wt^{-1} (glycine standard), while secondary amines doubled from 48 to 95 m$M\cdot$kg^{-1}. The net increase of all amines was 155 mM FAA$\cdot$kg dry wt^{-1}. Based on IC water, primary amines increased from 285 to 340 m$M\cdot$l^{-1} intracellular water, while proline rose from 23.4 to 47.5

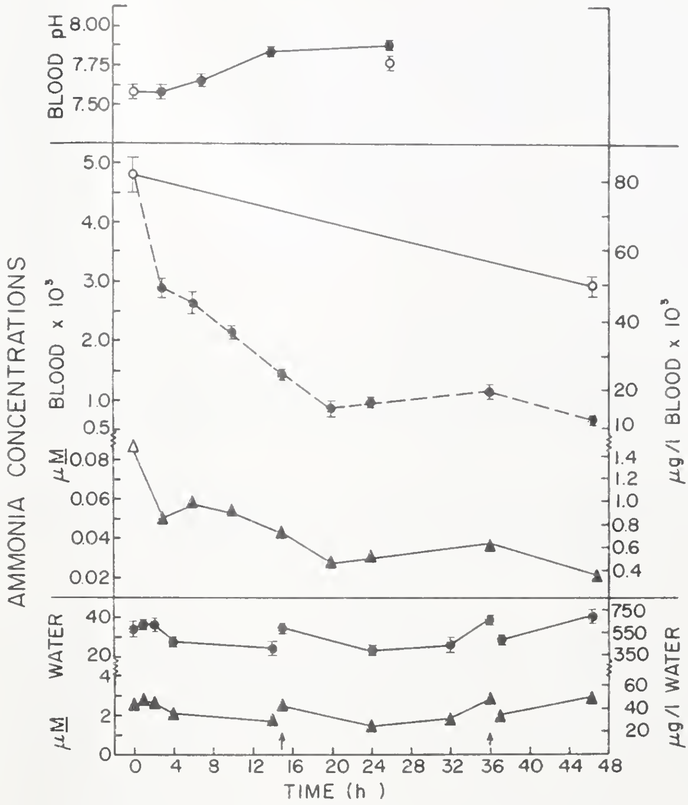
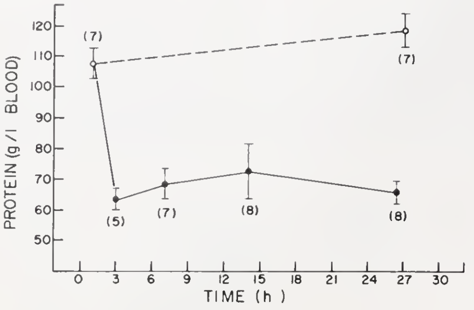


FIGURE 2. Change in concentrations of ammonia and pH values in blood of shrimp transferred from 0‰ to 24‰ (mean ± SE of 9 to 15 animals per interval). Total ammonia added to beakers at T_0 = 30 μM. Mean water values are given for comparison to blood concentrations. For both water and blood, circles = total ammonia ($NH_4^+ + NH_3$), ○ = 0‰, ● = 24 ‰; triangles = un-ionized ammonia (NH_3). NH_3 was calculated as described in text, pK'a = 9.33 for shrimp blood and ambient water at 24‰. Water pH constant at 8.23, blood pH beyond 26 h assumed to be 7.85.

$mM \cdot l^{-1}$; a total net gain in these two FAA groups of about 78 $mM \cdot l^{-1}$ intracellular water.

M. rosenbergii slowly acclimated to various salinities strongly regulated blood Na^+ concentrations in ambient waters from 0‰ to 24‰ (Fig. 6A). Animals hyperregulated Na^+ to an isotonic point of about 210 m*M* Na (≈14‰) and hyporegulated Na^+ between salinities of 14–28‰. Over an ambient Na^+ concentration of 5 (0‰) to 366 (24‰) m*M*, average blood sodium increased from 150 to 250 m*M*.

After a transfer from 0‰ to 24‰, blood Na^+ and total osmolarity, initially very hypotonic to the new medium, began to increase in 2–3 h. The rate of net sodium increase in blood over the first 7 h was 12.1 $mM \cdot h^{-1}$, and between 7 and 48 h only 1.2 $mM \cdot h^{-1}$ (Fig. 6B). Forty-eight h after the transfer there was still a large hypotonic gradient between blood and water Na^+ (285 and 366 m*M*, respectively; Fig. 6B). The blood equilibration value of 285 m*M* Na^+ after abrupt transfer was very close to 260 m*M* when shrimp were gradually acclimated to 24‰ (Fig. 6A).



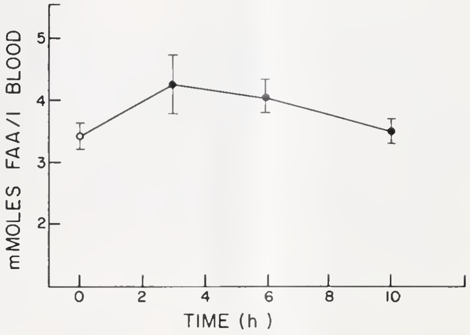


FIGURE 3. Concentrations of protein in the hemolymph of *M. rosenbergii* following a transfer from 0 ‰ to 24 ‰. Points are means ± SE, sample size in parenthesis. ○ = FW, ● = 24 ‰.

FIGURE 4. Concentrations of total primary amino acids in hemolymph of *M. rosenbergii* transferred from FW (○) to 24‰ (●). Points are means ± SE of 10–14 animals per time.

In contrast, total blood osmolarity increased 12.5 $\mathrm{mOsm \cdot l^{-1} \cdot h^{-1}}$ and was isosmotic to 24‰ (693 $\mathrm{mOsm \cdot l^{-1}}$) 20 h after transfer (Fig. 6B).

DISCUSSION

Although an essential ingredient of many organic compounds, ammonia is usually viewed as a potentially toxic molecule, excreted from organisms as anabolic requirements are exceeded by catabolic release from assimilated food and by turnover of endogenous nitrogen compounds. Having once left an organism, ammonia *per se* is not thought to re-enter the bodies of higher aquatic animals except in toxic situations. We have shown in this study that when the euryhaline shrimp *M. rosenbergii* is subjected to a hyperosmotic shock, external ammonia decreases during the first 20–24 h after transfer to saltwater. This loss of ambient ammonia cannot be accounted for by bacterial uptake or aerial diffusion to the atmosphere,

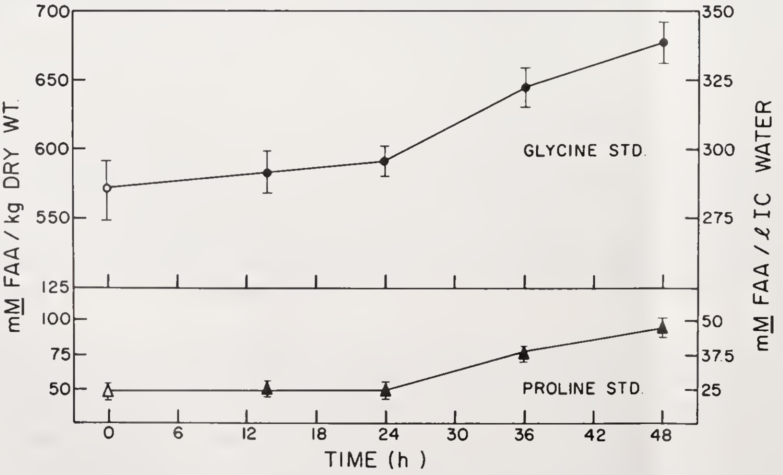


FIGURE 5. Concentrations of free amino acids in the tail muscle of *M. rosenbergii* acclimated to FW (open symbols) and following abrupt transfer to 24‰ (closed symbols) for 48 h.

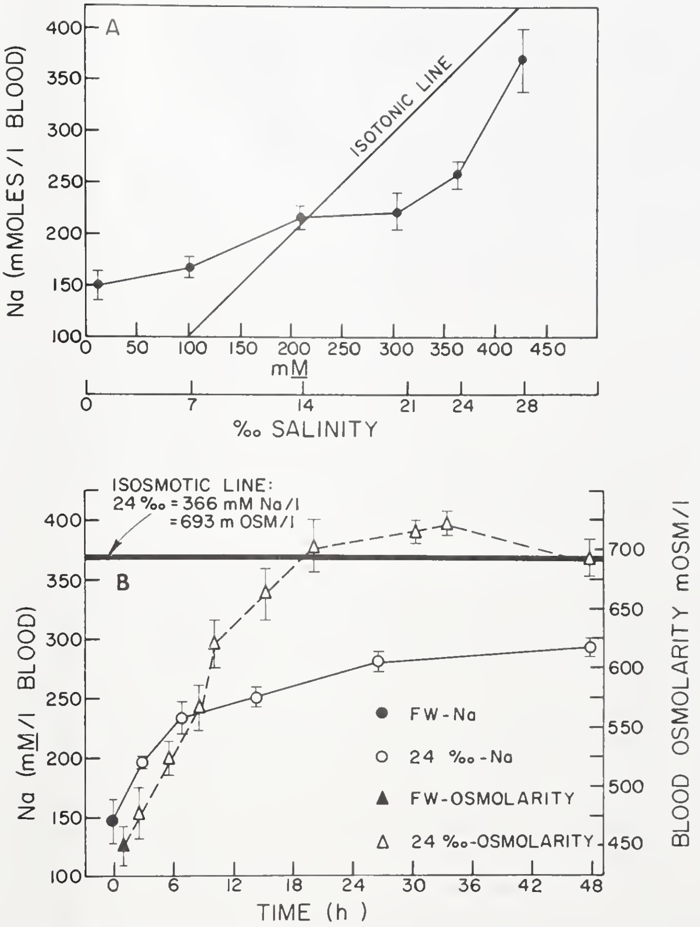


FIGURE 6. (A) Blood Na^+ concentrations in shrimp gradually acclimated to a specific salinity from 0‰ to 28‰. Animals were held at a given test salinity 48 h before sampling. Points are mean ± SE of at least eight animals per salinity. (B) Time course change in blood Na^+ and total osmolarity after an abrupt transfer from 0‰ to 24‰. Points are mean ± SE of data from 8–15 animals per interval.

and therefore indicates uptake by the animals themselves. This is the first report of ammonia uptake by higher aquatic animals, although several authors have suggested that possibility.

Maetz (1973) found that sodium influx to goldfish was inhibited by increased ammonia in the water, yet ammonia excretion was not. He concluded that an NH_4^+/NH_4^+ exchange was operative, but did not pursue its possible significance. During experiments to study the excretion of ammonia as NH_4^+ or NH_3 from marine invertebrates, Mangum *et al.* (1978) found that total ammonia of ambient water in vessels holding the clam *Rangia cuneata* declined from 5 to 2.44 m*M* during tests. Discounting loss by volatilization, they concluded that at least some ammonia permeated the body. Shaw (1960) reported the competitive inhibition of Na^+ influx into crayfish by NH_4^+ and postulated that this implied movement of the latter into animals. Thomas *et al.* (1976) reported enhanced growth of the gastropod *Biomphalaria glabrata* in alkaline water containing high concentrations of ambient ammonia up to 75 mg/l. They hypothesized that NH_3 might diffuse into snails and serve a function in $CaCO_3$ deposition and/or act as a Na^+ counter-ion once protonated.

Changes in ammonia excretion following a hyperosmotic transfer have been studied in numerous crustacea (Jeuniaux and Florkin, 1961; Krishnamoorthy and Srihari, 1973; Mangum *et al.*, 1976). Although rates typically decline, excretion remains positive. Why, then, have previous studies not found evidence of transitory ammonia uptake as we report? Apart from intrinsic differences in the osmoregulator biochemistry between species, variations in experimental protocol may be the main reasons. Acclimation of animals to high salinities for several days to weeks often precedes measurement of ammonia excretion (Sharma, 1966; Krishnamoorthy and Srihari, 1973; Mangum *et al.*, 1976), thus giving animals sufficient time to equilibrate to a situation that may temporarily require ambient ammonia. In addition, samples may not have been taken frequently enough to observe temporary trends in ammonia fluxes, or excretion may have been based merely on two ammonia determinations over some period of time, so that the rate calculated is an average of trends during the interval (Needham, 1957; Sharma, 1966; Krishnamoorthy and Shriahari, 1973; Mangum *et al.*, 1976).

Possible osmoregulatory function of ammonia uptake: Na^+ regulation

Exogenous ammonia could have two possible osmoregulatory functions during acclimation to high salinity. The first deals with the poorly understood and apparently complicated branchial Na^+/NH_4^+ exchange in fish and crustacea (Kirschner *et al.*, 1973; Maetz *et al.*, 1976; Kirschner, 1979; Evans, 1980). Evidence indicates that freshwater aquatic organisms keep their blood Na^+ concentration hypertonic to ambient water by active uptake of external Na^+, in part via exchange for blood NH_4^+ and/or H^+. A number of recent studies indicate that seawater teleosts and elasmobranchs also possess Na^+/NH_4^+ exchange mechanisms (Payan and Maetz, 1973; Evans, 1975, 1977, 1980). Since seawater fishes maintain blood Na^+ concentrations hypotonic to the external environment, these exchange mechanisms impose an increased Na^+ load on the animal in addition to that incurred by drinking for water balance. Evans (1980) notes that the size of this load varies with species studied to date and may become appreciable in those organisms that maintain a branchial transepithelial potential close to that of the equilibrium potential for Na^+.

Na^+ and NH_4^+ probably are exchanged across the branchial epithelium of freshwater-acclimated *M. rosenbergii.* A rapid transfer from FW (5 m*M* Na^+) to 24‰ (366 m*M*) immediately establishes a large hypotonic gradient between blood Na^+ (150 m*M* in FW) and the new medium. We tentatively suggest that during subsequent acclimation to 24‰, the direction of the Na^+/NH_4^+ pumps changes such that internal Na^+ is extruded in exchange for exogenous NH_4^+ (Na^+ blood/NH_4^+ seawater). Although blood Na^+ increases to about 285 m*M*, it remains strongly hypotonic to the medium. It seems logical that an active pump, functioning to extrude Na^+, would mitigate the increased diffusional influx. This putative reversal of Na^+/NH_4^+ exchange may be an important mechanism allowing the animal to hyporegulate blood Na^+ some 85 m*M* below ambient seawater.

Although we are aware of no previous work on the reversibility of the Na^+/NH_4^+ pump, De Renzis and Bornancin (1977) have demonstrated the existence of Cl^-/HCO_3^- ATPase in fish, and Kormanik and Evans (1979) have provided evidence for reversal of such a Cl^-/HCO_3^- pump in high salinities. The Cl^-/HCO_3^- exchange system of fish gill epithelia are as poorly understood as Na^+/NH_4^+ exchange, but most evidence indicates that in freshwater fish and crustacea, part of the Cl^- influx is coupled to efflux of HCO_3^- (De Renzis and Maetz, 1973; Ehrenfeld, 1974; Maetz *et al.*, 1976; Kirschner, 1979). In the seawater acclimated Gulf toadfish, however, Kormanik and Evans (1979) found that external HCO_3^- stimulated undirectional Cl^- efflux with a K_m for HCO_3^- similar to normal seawater concentrations. This suggests that internal Cl^-, which is strongly hyporegulated, is exchanged for external HCO_3^-.

The proposed reversal of Na^+/NH_4^+ exchange during hyporegulation by *M. rosenbergii* in high salinity relies on the dual structure of ammonia. Active excretion of Na^+ can be linked to an ammonia cycle composed of exchange transport of exogenous NH_4^+ and back diffusion of NH_3 down a significant concentration gradient. In the current experiments, uptake of exogenous ammonia may greatly exceed ammonia excretion (diffusion) during the first 22–24 h, resulting in a net decrease in ammonia in ambient water. This is the period of greatest change in blood Na^+ concentrations, and may reflect a more extensive effort to control rapid Na^+ influx than is required after equilibration (Fig. 6B). Net ammonia gained in this period could be used in a second osmoregulatory function involving synthesis of intracellular FAA (see following section). During further acclimation to seawater (*i.e.* later than 24 h after transfer), net excretion may exceed uptake, possibly because of a less intense need to regulate Na^+ and/or because of other regulatory exchange mechanisms (such as Na^+ blood/K^+ seawater) responding to the initial hyper-saline transfer.

The putative reversal of Na^+/NH_4^+ exchange also might continue hyporegulating blood Na^+ as a permanent feature of salt water acclimation. The NH_4^+ uptake-NH_3 diffusion cycle of this tentative model depends on intracellular branchial pH and total ammonia concentrations. Together, both factors determine the magnitude of the NH_3 gradient from cell to water. The pH difference *per se* probably will not favor diffusion outward, since ambient pH of our experiments was 8.28 and that of the blood about 7.62. IC pH of branchial cells would be a more likely factor, since this is the fluid first exchanged between the animal and the medium. For crustacea, IC pH in muscle may be 0.5–0.8 units lower than blood (Caldwell, 1954, 1958; Carter, 1972). Therefore, IC branchial pH might be 1.0 units less than the water of our tests, resulting in a 10-fold decrease in NH_3 on the acid IC side. Mangum and Towle (1977) depict a similar situation in *Callinectes sapidus* in high salinity, and suggest NH_3 diffuses from water to blood. However, the second factor to consider in anticipating the direction of NH_3 diffusion is total ammonia. In this regard, blood ammonia is not an appropriate measure as used in some calculations (Mangum and Towle, 1977; Armstrong *et al.*, 1978). Again, the branchial IC concentrations are the important pools of ammonia in discussions of Na^+/NH_4^+ exchange, and IC ammonia may be quite high.

Osmoregulatory studies have provided some fortuitous measures of ammonia in whole-body homogenates and isolated tissues of crustaceans, ranging from 2.2 to as high as 53 m*M* ammonia in IC water (recalculated from data of Gilles and Schoffeniels 1969a, b; Vincent-Marique and Gilles, 1970; Siebers *et al.*, 1972; assuming an average total body water content of 74% of wet weight and IC water equal to 78% of total body water). Blood ammonia, however, ranges from 0.05 to 1.0 m*M* (Gerard and Gilles, 1972; Siebers *et al.*, 1972; Mangum *et al.*, 1976). Thus, there is a 50-fold difference between extremes of the IC and blood ranges.

In molluscs, hemolymph ammonia is about 0.8 m*M* (Bartberger and Pierce, 1976; Strange and Crowe, 1979), while IC ammonia is about 10 to 20 m*M* and may be higher (recalculated from data of Shumway and Abbott, 1977, and Livingstone *et al.*, 1979; water content = 76% of wet wt and IC water assumed to be 78% of this value). From our tests, data on quantities of ammonia lost following the hypersaline transfer have been calculated as concentrations per liter IC water (*see* "Materials and Methods"). Based on these calculations, IC ammonia could have increased by 7.8 m*M* 24 h after transfer. Add to this the quantities already present and that gained from blood, and IC ammonia for the entire animal could easily exceed 10 m*M*, as discussed previously for other invertebrates. (Since blood ammonia declined significantly after the hyperosmotic transfer (Fig. 2), extracellular fluids were not considered a probable sink for ambient ammonia lost during the first 24 h.) Again, considering that natural concentrations of ambient ammonia range from tenths μM to usually no more than 2-10 μM, the gradient of NH_3 from branchial cells to water may be several orders of magnitude, despite potentially unfavorable pH gradients.

Thus, the long-term (greater than 24 h) osmoregulatory response after a hyperosmotic transfer, and subsequent active hyporegulation of blood Na^+, could include an exchange for ambient NH_4^+, a portion of which continuously dissociates and rapidly diffuses back out the gills down a large concentration gradient. This in turn would result in a net excretion of ammonia, thereby preventing toxic buildup within the animal's body. Uptake of exogenous NH_4^+ in exchange for actively excreted Na^+ would compliment the model of Cl^-/HCO_3^- reversal proposed by Kormanik and Evans (1979). Once transported from water to cell, NH_4^+ would dissociate to NH_3, diffuse out (diffusion of NH_3 is the principle mechanism of ammonia excretion in blue crabs; Kormanik and Cameron, 1980), and leave H^+ to combine with the HCO_3^- exchanged for hyporegulated blood Cl^-. The uncharged H_2CO_3 could diffuse as is, or be converted to CO_2 and H_2O via carbonic anhydrase. Our model of reversed Na^+/NH_4^+ exchange is obviously tentative and awaits further experimental verification. A first study would measure ammonia and sodium fluxes during and after acclimation to high salinity.

Ammonia uptake: free amino acid synthesis

As discussed previously, ambient ammonia lost in the first 24 h after transfer could increase IC concentrations by as much as 7.8 m*M*. This rise in IC ammonia could stimulate GDH and simultaneously inhibit oxidative reactions of the Krebs cycle to maximize FAA production, as noted by Gilles and Schoffeniels (1969b). However, our data on tail muscle FAA concentrations show only a small increase within 24 h (Fig. 5). This suggests that either ambient ammonia does not contribute substantially to FAA synthesis, or that ambient ammonia is not distributed equally to the IC fluids of all tissues: Certain tissues such as nerves and gill epithelia may have to regulate cell volume rapidly, and hence may preferentially accumulate ambient ammonia for synthesis of amino acids.

ACKNOWLEDGMENTS

The authors thank Dr. R. Burau, U. C. Davis, for help with analyses of blood cations. This work was supported by a grant from the State of California to the U.C. Davis Aquaculture Group (A.W.K.), NSF grant PCM 77-07119 (J.H.C.), and a grant from the Graduate School, University of Washington (D.A.A.). We appreciate use of facilities at Friday Harbor Marine Laboratory, University of Washington, for work on the manuscript.

LITERATURE CITED

Armstrong, D. A., M. J. Stephenson, and A. W. Knight. 1976. Acute toxicity of nitrite to larvae of the giant Malaysian prawn, *Macrobrachium rosenbergii*. *Aquaculture* **9:** 39–46.

Armstrong, D., D. Chippendale, A. Knight, and J. Colt. 1978. Interaction of ionized and un-ionized ammonia on short-term survival and growth of larvae of the prawn, *Macrobrachium rosenbergii*. *Biol. Bull.* **152:** 15–31.

Bartberger, C. A., and S. Pierce. 1976. Relationship between ammonia excretion rates and hemolymph nitrogenous compounds of an euryhaline bivalve during low salinity acclimation. *Biol. Bull.* **150:** 1–14.

Binns, R. J., and A. Peterson. 1969. Nitrogen excretion by the spiny lobster *Jasus edwardsi* (Hutton): the role of the antennal gland. *Biol. Bull.* **136:** 147–153.

Bishop, S. 1976. Nitrogen metabolism and excretion: regulation of intracellular amino acid concentrations. Pp. 414–431 in M. Wiley, Ed., *Estuarine processes. Vol. 1: Uses, stresses and adaptation to the estuary*. Academic Press, New York.

Boktor, F. N. 1971. An improved method for colorimetric determination of proline. *Anal. Biochim.* **43:** 66–70.

Bowlus, R. D., and G. N. Somero. 1979. Solute compatibility with enzyme function and structure: rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. *J. Exp. Zool.* **208:** 137–152.

California, State of, Resources Agency. 1976. Delta-Suisan Bay ecological studies, 1973, 1974, and water quality surveillance program 1976. Vol. I. Sacramento-San Joaquin Delta. 158 pp.

Caldwell, P. 1954. An investigation of the intracellular pH of crab muscle fibers by means of micro-glass and micro-tungsten electrodes. *J. Physiol.* **126:** 169–180.

Caldwell, P. 1958. Studies on the internal pH of large muscle and nerve fibers. *J. Physiol.* **142:** 22–62.

Campbell, J. 1973. Nitrogen excretion. Pp. 279–316 in C. L. Prosser, Ed., *Comparative animal physiology*. W. B. Saunders Co., New York.

Carter, N. W. 1972. Intracellular pH. *Kidney Int.* **1:** 341–346.

Colt, J., and G. Tchobanoglous. 1976. Evaluation of the short-term toxicity of nitrogenous compounds to channel catfish, *Ictalurus punctatus*. *Aquaculture* **8:** 209–224.

DeRenzis, G., and J. Maetz. 1973. Studies on the mechanism of choride absorption by the goldfish gill: relation with acid-base regulation. *J. Exp. Biol.* **59:** 339–358.

DeRenzis, G., and M. Bornancin. 1977. A Cl/HCO_3-ATPase in the gills of *Carassius auratus*: its inhibition by thiocyanate. *Biochem. Biophys. Acta* **467:** 192–207.

Ehrenfeld, J. 1974. Aspects of ionic transport mechanisms in crayfish *Astacus leptodactylus*. *J. Exp. Biol.* **61:** 57–70.

Evans, D. H. 1975. Ionic exchange mechanisms in fish gills. *Comp. Biochem. Physiol.* **51A:** 491–495.

Evans, D. H. 1977. Further evidence for Na/NH_4 exchange in marine teleost fish. *J. Exp. Biol.* **70:** 213–220.

Evans, D. H. 1980. Kinetic studies of ion transport by fish gill epithelium. *Am. J. Physiol.* **7:** 224–230.

George, M. J. 1969. Genus *Macrobrachium* (Bates 1968). *Central Mar. Fish. Res. Instit., India Bull.* **14:** 179–204.

Gerard, J. F., and R. Gilles. 1972. The free amino-acid pool in *Callinectes spidus* (Rathbun) tissues and its role in the osmotic intracellular regulation. *J. Exp. Mar. Biol. Ecol.* **10:** 125–136.

Gilles, R. 1975. Mechanisms of ion and osmoregulation. Pp. 259–347 in O. Kinne, Ed., *Marine ecology*, Vol. II, Part I. John Wiley and Sons, New York.

Gilles, R. 1979. Intracellular organic osmotic effectors. Pp. 111–156 in R. Gilles, Ed., *Mechanisms of osmoregulation in animals*. John Wiley and Sons, New York.

Gilles, R., and E. Schoeffeniels. 1969a. Isomotic regulation in isolated surviving nerves of *Eriochier sinensis* Milne Edwards. *Comp. Biochem. Physiol.* **31:** 927–939.

Gilles, R., and E. Schoffeniels. 1969b. Metabolism of arabinose in the ventral nerve cord of the lobster *Homarus vulgaris* (M. Edw.). *Comp. Biochem. Physiol.* **29:** 1145–1152.

Hutchinson, G. E. 1957. *A treatise on limnology. I. Geography, physics, and chemistry*. John Wiley and Sons, New York. 1015 pp.

Jeuniaux, Ch., and M. Florkin. 1961. Modification de l' excrétion azotée du crabe chinois au cours de l' adaptation osmotique. *Arch. Int. Physiol. Biochim.* **69**: 385–386.

Johnson, D. S. 1967. Some factors influencing the distribution of freshwater prawns in Malaya. *Proc. Symp. Crus. Mar. Biol. Assoc. India* **1**: 418–433.

Kerley, D. E., and A. W. Pritchard. 1967. Osmotic regulation in the crayfish *Pacifastacus leniusculus*, stepwise acclimated to dilutions of sea water. *Comp. Biochem. Physiol.* **20**: 101–113.

Kirschner, L. B. 1979. Control of the extracellular fluid osmolarity: control mechanisms in crustaceans and fishes. Pp. 157–222 in R. Gilles, Ed., *Mechanisms of osmoregulation in animals*. John Wiley and Sons, New York.

Kirschner, L., L. Greenwald, and T. Kerstetter. 1973. Effect of amiloride on sodium transport across body surface of freshwater animals. *Am. J. Physiol.* **224**: 832–837.

Kormanik, G., and D. Evans. 1979. HCO_3-stimulated Cl efflux in the Gulf toadfish acclimated to seawater. *J. Exp. Zool.* **208**: 13–16.

Kormanik, G., and J. Cameron. 1980. Ammonia excretion in the blue crab: diffusion and not Na^+/NH_4^+ exchange. *Physiologist* **23**: 175.

Krishnamoorthy, R. V., and K. Srihari. 1973. Changes in the excretory patterns of the freshwater field crab *Paratelphresa hydrodromous* upon adaptation to higher salinities. *Mar. Biol.* **21**: 341–348.

Lang, M. A., and H. Gainer. 1969. Isosmotic intracellular regulation as a mechanism of volume control in crab muscle fibers. *Comp. Biochem. Physiol.* **30**: 445–456.

Lee, C., and J. Boda. 1977. Dissolved amino acids in the equatorial Pacific, the Sargasso Sea and Biscayne Bay. *Limnol. Oceanogr.* **22**: 502–510.

Livingstone, D., J. Widdows, and P. Fieth. 1979. Aspects of nitrogen metabolism of the common mussel *Mytilis edulis*: adaptation to abrupt and fluctuating changes in salinity. *Mar. Biol.* **53**: 41–55.

Lowry, O., N. Rosebrough, A. Farr, and R. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

Maetz, J. 1973. Na^+/NH_4^+, Na^+/H^+ exchanges and NH_3 movement across the gill of *Carassius auratus*. *J. Exp. Biol.* **58**: 255–275.

Maetz, J., P. Payan, and G. DeRenzis. 1976. Controversial aspects of ionic uptake in freshwater animals. Pp. 77–92 in S. Davis, Ed., *Perspectives in experimental biology, Vol. I.* Pergamon Press, New York.

Mangum, C. P., and D. Towle. 1977. Physiological adaptation to unstable environments. *Amer. Sci.* **65**: 67–75.

Mangum, C. P., S. Silverthorn, J. Harris, D. Towle, and A. Krall. 1976. The relationship between blood pH, ammonia excretion and adaptation to low salinity in the blue crab *Callinectes sapidus*. *J. Exper. Zool.* **195**: 129–136.

Mangum, C. P., J. Dykens, R. Henry, and E. Polites. 1978. The excretion of NH_4^+ and its ouabain sensitivity in aquatic annelids and molluscs. *J. Exp. Zool.* **203**: 151–157.

Needham, A. E. 1957. Factors effecting nitrogen excretion in *Carcinides maenas* (Pennant). *Physiol. Comp. Oecol.* **4**: 209–239.

Nelson, S., A. Knight, and H. Li. 1977. The metabolic cost of food utilization and ammonia production by juvenile *Macrobrachium rosenbergii* (Crustacea: Palomonidae). *Comp. Biochem. Physiol.* **57A**: 67–72.

North, B. 1975. Primary amines in California coastal waters: utilization by phytoplankton. *Limnol. Oceanogr.* **20**: 20–27.

Payan, P., and J. Maetz. 1973. Branchial sodium transport mechanisms in *Scyliorhinus canicula*: evidence for Na^+/NH_4^+ exchanges and for a role of carbonic anhydrase. *J. Exp. Biol.* **58**: 487–502.

Prosser, C. L. 1973. Water: Osmotic balance; hormonal regulation. Pp. 1–78 in C. L. Prosser, Ed., *Comparative animal physiology*. W. B. Saunders, Philadelphia.

Sandifer, P., J. Hopkins, and T. Smith. 1975. Observations on salinity tolerance and osmoregulation in laboratory-reared *Macrobrachium rosenbergii* post-larvae (Crustacea: Caridea). *Aquaculture* **6**: 103–114.

Schoffeniels, E. 1976. Adaptations with respect to salinity. *Biochem. Soc. Symp.* **41**: 179–204.

Sharma, M. L. 1966. Studies on the changes in the pattern of nitrogenous excretion of *Orconectes rusticus* under osmotic stress. *Comp. Biochem. Physiol.* **19**: 681–690.

Shaw, J. 1960. The absorption of sodium ions by the crayfish *Astacus pallipes* Lereboullet. III. The effect of other cations in the external solution. *J. Exp. Biol.* **37**: 548–556.

Shumway, S., and P. G. Abbott. 1977. The effect of fluctuating salinity on the concentrations of free amino acids and ninhydrin-positive substances in the adductor muscles of eight species of bivalve molluscs. *J. Exp. Mar. Biol. Ecol.* **29:** 131–150.

Siebers, D., C. Lucu, K.-R. Spealing, and K. Eberlein. 1972. Kinetics of osmoregulation in the crab *Carcinus maenas*. *Mar. Biol.* **17:** 291–303.

Sindermann, C., Ed. 1977. *Diseases of North American aquaculture animals*. Elsevier Press, New York. 329 pp.

Solorzano, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.* **14:** 799–801.

Spaargaren, D. 1972. Determination of the extracellular volume in the shrimp *Crangon crangon* (L.). *Comp. Biochem. Physiol.* **43A:** 843–848.

Stanier, R., M. Doudoroff, and E. Adelberg. 1970. *The microbial world*. Prentice-Hall, Inc., New York. 873 pp.

Strange, K., and J. Crowe. 1979. Acclimation to successive short term salinity changes by the bivalve *Modiolus demissus*. II. Nitrogen metabolism. *J. Exp. Zool.* **210:** 227–235.

Thomas, J., M. Powles, and R. Lodge. 1976. The chemical ecology of *Biomphalaria glabrata*: the effects of ammonia on the growth rate of juvenile snails. *Biol. Bull.* **151:** 386–397.

Vincent-Marique, C., and R. Gilles. 1970. Modification of the amino acid pool in blood and muscle of *Eriocheir sinensis* during osmotic stress. *Comp. Biochem. Physiol.* **35:** 479–485.

Reference: *Biol. Bull.* **160:** 366–375. (June, 1981)

PROTEIN SYNTHESIS IN THE POLAR LOBE AND LOBELESS EGG OF *ILYANASSA OBSOLETA*

J. R. COLLIER

Biology Department, Brooklyn College, Brooklyn, N. Y. 11210

ABSTRACT

The relative rates of protein synthesis in the lobeless egg and polar lobe of *Ilyanassa obsoleta* were measured and found to be the same. Two-dimensional electrophoresis of ^{35}S-methionine *in vivo* labeled polypeptides extracted from the *Ilyanassa* egg and its isolates resolved the same set of polypeptides from the egg, lobeless egg, and isolated polar lobe. For those peptides detected it was concluded that (1) the lobeless egg and the polar lobe contain the same set of mRNAs, (2) these mRNAs are not differentially translated in either of these isolates, (3) the polar lobe does not regulate the synthesis of these proteins, either quantitatively or qualitatively, during early cleavage, and (4) the peptides synthesized in the isolated polar lobe were translated from mRNAs produced during oogenesis.

It was shown by one-dimensional electrophoresis of ^{35}S-methionine-labeled basic peptides that the egg, lobeless egg, and isolated polar lobe synthesized, at approximately the same rate, two variants of H1 histone, histones H2A, H2B, H3, and H4, and high mobility group peptides 14 and 17. Because these peptides were synthesized in the isolated polar lobe it was concluded that they were translated from maternal mRNAs.

INTRODUCTION

The significance of the role played by egg cytoplasm in determination and differentiation was established in the early embryological literature (Wilson, 1928). The isolation of the polar lobe and the separation of blastomeres by Crampton (1896) and Clement (1952, 1956) of the egg of *Ilyanassa obsoleta*, the common mud snail of the North American Atlantic coast, demonstrated the importance of the localization of specific kinds of cytoplasm within this egg and the selective distribution of egg cytoplasm to the early blastomeres.

Clement and Tyler (1967) demonstrated that the isolated polar lobe, a vegetal merogone lacking a nucleus, can synthesize proteins. The influence of removing the polar lobe on the embryo's synthesis of individual polypeptides has been investigated recently (Newrock and Brandhorst, 1979; Collier and McCarthy, 1979; McCarthy and Collier, 1980).

The present investigation of protein synthesis during early cleavage was prompted by the question of whether the expression of structural genes is involved in the early determinative events of this spiralian egg. Relative rates of protein synthesis by different regions of the egg were measured by determining the incorporation of radioactive amino acids into acid-precipitable proteins and by two-dimensional electrophoresis of *in vivo* labeled peptides. The contribution of mito-

---

Received 29 January 1981; accepted 6 March 1981.

Abbreviations: HMG, high mobility group; NP-40, Tergito, type NP-40; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

chondria to protein synthesis was assessed, and the synthesis of histones and high mobility group peptides was determined in the *Ilyanassa* egg and the polar lobe.

MATERIALS AND METHODS

Maintenance of snails and rearing of embryos

Mud snails collected at Plumb Beach in Brooklyn, New York, were kept in a 20-gal tank of recirculating sea water. Fed one clam on alternate days, they deposited a few hundred fresh egg capsules daily. Eggs and embryos were reared in filtered sea water from the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, with 200 μg/ml each of streptomycin and pencillin added. Polar lobes were isolated in sea water low in calcium and magnesium, as previously described (Collier, 1975). Embryos were reared at 19°C and staged by time of development from the two-cell stage.

All groups of embryos designated for direct comparison, *e.g.* normal and lobeless embryos, were obtained from a common pool of eggs.

Labeling of proteins

Proteins were labeled *in vivo* by rearing embryos for 4 h in 120 μCi/ml of L(^{35}S)-methionine (New England Nuclear, 800–1100 Ci mmol^{-1}). Radioactive methionine was dried *in vacuo* in a small glass vial to which was added 100–200 embryos in 0.3 ml of sea water containing penicillin and streptomycin. This vial was continuously rotated in a Rollerdrum (New Brunswick Scientific Co.) for 4 h at room temperature (24°C).

For the experiments with cycloheximide, embryos were labeled by incubation in 200–500 μCi/ml of ^{3}H-leucine (53.8 Ci mmol^{-1}, New England Nuclear) by the procedure described above.

Measurement of uptake and incorporation of amino acids

Labeled embryos or polar lobes were thoroughly rinsed with sea water, counted, placed in 0.2 ml of 2% SDS (sodium dodecyl sulfate) and heated at 100°C for 3 min. One portion of the SDS solubilized protein was counted in Bray's (1960) solution to determine the total radioactivity in the embryos. Another portion was precipitated onto a Millipore filter from cold 10% TCA (trichloroacetic acid), washed with 5% TCA, and counted in a scintillation spectrometer by dissolving the filter in Bray's solution.

Sample preparation for two-dimensional electrophoresis

Embryos labeled with ^{35}S-methionine were washed twice in calcium-magnesium-free sea water; suspended in 0.05 ml of this sea water; mixed vigorously for 30 s in an equal volume of homogenizing solution (0.01 *M* EDTA (ethylenediaminetetraacetic acid), 0.1 *M* methionine, 1.0% SDS, and 10% mercaptoethanol); and then heated to 100°C for 3 min. This solubilized homogenate was cooled, frozen, and lyophilized to dryness. The lyophilized homogenate was dissolved in 0.1 ml of a sample buffer containing 8.5 *M* urea (Schwarz-Mann Ultrapure), 2.0% NP-40 (Tergito, type NP-40; Sigma Chemical Co.), 0.1 *M* dithiothreitol, 0.05 *M* EDTA, 1.2% pH 3–10 ampholyte, and 1.6% pH 5–7 ampholyte (all ampholytes were obtained from BioRad Laboratories). The sample was then stored at −70°C.

Electrophoresis

Two-dimensional electrophoresis was carried out as described by O'Farrell (1975) with the following modifications: the isoelectric focusing gels contained 8.5 *M* urea and 2.8% ampholytes (1.2% pH 3–10 and 1.6% pH 5–7 Biolyte obtained from BioRad Laboratories). The gels were polymerized in 10.5 cm × 0.3 cm Lucite tubes that were held at 25°C during electrophoresis for 8400 volt-hours.

Second dimension electrophoresis of the first dimension gels was on discontinuous SDS slab gels, 16.0% acrylamide separating gels, and 5.4% acrylamide stacking gels, as described by Laemmli (1970), except that the gels contained 0.15% instead of 0.8% bisacrylamide. The dried gels were prepared for fluorography as described by Garrells (1979) and exposed to Kodak XR-5 X-ray film.

The volume of all samples used for electrophoresis was adjusted so that each sample designated for comparison contained the same mass of protein. Variation in radioactivity of the samples was compensated for by the exposure time of the autoradiogram.

Electrophoresis of histones

Embryos labeled with ^{35}S-methionine as described above were washed in several changes of sea water and solubilized by heating at 100°C for 3 min in 0.0625 *M* Tris, pH 6.8, containing 2% SDS and 50 m*M* dithiothreitol. Bromophenol Blue tracking dye was added to the sample, which was then frozen until used. The frozen samples were thawed and loaded onto a discontinuous acrylamide slab gel as formulated by Laemmli (1970), but modified to contain 0.15% instead of 0.8% bisacrylamide. The separating gel contained 16% acrylamide. Electrophoresis was continued until the tracking dye moved off of the gel.

The position of the histones on the autoradiograph was established by co-electrophoresis of ^{35}S-methionine labeled basic proteins with histones extracted from isolated nuclei of *Ilyanassa* embryos. The slab gel was stained with Coomassie Blue and then dried. The dried gel and a sheet of X-ray film were held in registry while both were notched several times along two sides. The gel and film were pinned together and the film was exposed to the gel for the required time. After the film had been developed this marking system provided a precise matching of the autograph with the stained gel, and obviated mismatching between the position of the stained histones (Fig. 2D) and radioactive bands on the autoradiograph from changes in the gel dimension during processing.

RESULTS

Mitochondrial protein synthesis

Generally, mitochondria are not expected to make a major contribution to polypeptide synthesis. However, the relatively low level of protein synthesis during early cleavage and the small amount of cytoplasm in the polar lobe require an assessment of mitochondrial protein synthesis. Accordingly, the effect of cycloheximide on protein synthesis during early cleavage was investigated.

Four-cell embryos were pulsed for 1 h with 200 μCi/ml of ^{3}H-leucine, washed with sea water, and transferred to varying concentrations of cycloheximide for the

TABLE I

Cycloheximide inhibition of protein synthesis during early cleavage. Leucine incorporation into protein as CPM per embryo

	Cycloheximide concentration micrograms per ml			
Time, min	0	100	200	400
60	230	257	373	327
	398	206	361	318
	441	155	206	327
Mean	356	206	313	313
	***†	***	NS	NS
120	581	570	418	393
	515	504	375	320
	548	561	333	261
Mean	548	545	375	324

† ***, $P < 0.01\%$; NS, $P > 0.05\%$ for significance of differences between means for treatment at 60 and 120 min.

times indicated in Table I. After 1 h 400 μg/ml of cycloheximide, a differential inhibitor of non-mitochondrial protein synthesis (Schatz and Mason, 1974), inhibited 96.5% of the incorporation of leucine into protein. Thus, little, if any, intramitochondrial protein synthesis occurred during early cleavage.

Relative rates of polypeptide synthesis in the egg, lobeless egg, and polar lobe

Table II shows the results of an experiment in which eggs were pre-loaded with ^{35}S-methionine for 1.5 h before the trefoil stage, removed from precursor, and delobed immediately. The total uptake of ^{35}S-methionine and its incorporation into protein was then determined for the egg, lobeless egg, and polar lobe. This experiment avoids the problem of differential uptake of precursor by any of the isolates, and measures the precursor's compartmentation into the lobeless egg and polar lobe. Table II, Part A shows that 77.1% of the total ^{35}S-methionine was partitioned into the lobeless egg and 16.9% into the polar lobe; similarly, Table II, Part B shows that these parts of the egg account for 76.8% and 12.9%, respectively, of the ^{35}S-methionine incorporated into protein. These distributions are not significantly different ($F(1, 4) = 1.58$, $P > 0.05$) from the 84.2% of the volume of non-yolk cytoplasm in the lobeless egg and 16.5% in the polar lobe (Collier, 1957). From this distribution of radioactive amino acids, I infer that the egg's amino acid pool, a quantity not directly measurable in a small number of eggs or isolates, is distributed into the lobeless egg and the polar lobe in proportion to their cytoplasmic volume. Accordingly, the ^{35}S-methionine incorporation into protein, insofar as it depends on the size of the amino acid pool, should be proportional to the cytoplasmic volume of the lobeless egg and the polar lobe. The data on the incorporation of radioactivity into protein per unit of cytoplasm (Table II, Part C) shows that this is the case. The measured values for this incorporation are not significantly different ($F(2, 6) = 2.23$, $P > 0.05$) for the egg, lobeless egg, or polar lobe. Thus, the egg and its isolates are synthesizing polypeptides at the same relative rates.

Electrophoresis of labeled polypeptides

Whether the average relative rate of protein synthesis, as measured by the incorporation of ^{35}S-methionine into acid-precipitable proteins, accurately reflected

TABLE II

Uptake and incorporation of ^{35}S-methionine

	Radioactivity and per cent distribution of radioactivity*				
		Lobeless egg		Polar lobe	
	Egg CPM	CPM	%	CPM	%
A. Total radioactivity:	159,003	118,677	74.6	31,240	19.6
	172,277	130,461	75.7	31,916	18.5
	125,532	101,730	81.0	15,808	12.6
Mean %			77.1		16.9
B. Acid precipitable CPM	33,414	27,251	81.6	5,424	16.2
	40,956	29,012	70.8	5,356	13.1
	26,073	20,341	78.0	2,436	9.4
Mean %			76.8		12.9
C. CPM × 10^7 incorporated per mm^3 cytoplasm:	2.5	2.4		2.5	
	3.1	2.6		2.4	
	2.0	1.8		1.1	
Mean	2.5	2.3		2.0	

* Significance of differences among groups was determined by an analysis of variance; F-distribution, degrees of freedom and *P* values are in the text.

the rate of synthesis of individual peptides was determined by separating polypeptides by two-dimensional electrophoresis on acrylamide gels. For these studies, ^{35}S-methionine was used because of its high specific activity and the high energy of its beta emission. These features made possible the use of a small number of embryos and relatively short exposure times for the autoradiographs. In Figure 1 are autofluorographs of two-dimensional gels of peptides extracted from eggs, lobeless eggs, and isolated polar lobes labeled *in vivo* from the two- to the twelve-cell stage with ^{35}S-methionine. These gels were loaded with the same amount of total cytoplasmic protein, *i.e.* total protein minus yolk protein. Differences in radioactivity loaded onto each gel were compensated for by the length of exposure of the autoradiographs. Comparing the position, size, and density of spots among these autofluorographs (Fig. 1) shows that the egg and its isolates synthesized the same set of peptides at the same relative rate of accumulation.

Synthesis of histones and high mobility group peptides

The two-dimensional electrophoresis of peptides described above did not resolve basic peptides. However, histones labeled *in vivo* with ^{35}S-methionine in normal eggs, lobeless eggs, and isolated polar lobes were resolved by one-dimensional electrophoresis on SDS gels, as shown in Figures 2A, B, and C. These fluorographs show the incorporation of methionine into two variants of H1 histones as well as into other histones, of which H2A and H2B are not individually resolved.

Densitometric scans of the fluorographs of histones in Figures 2A, B, and C were made, and the area under the scans was determined as a measure of the accumulation of radioactivity into the H4 histone for each region of the egg. Histone H4 was selected as that histone least likely to be contaminated by traces of non-histone peptides that had incorporated the high specific activity ^{35}S-methionine

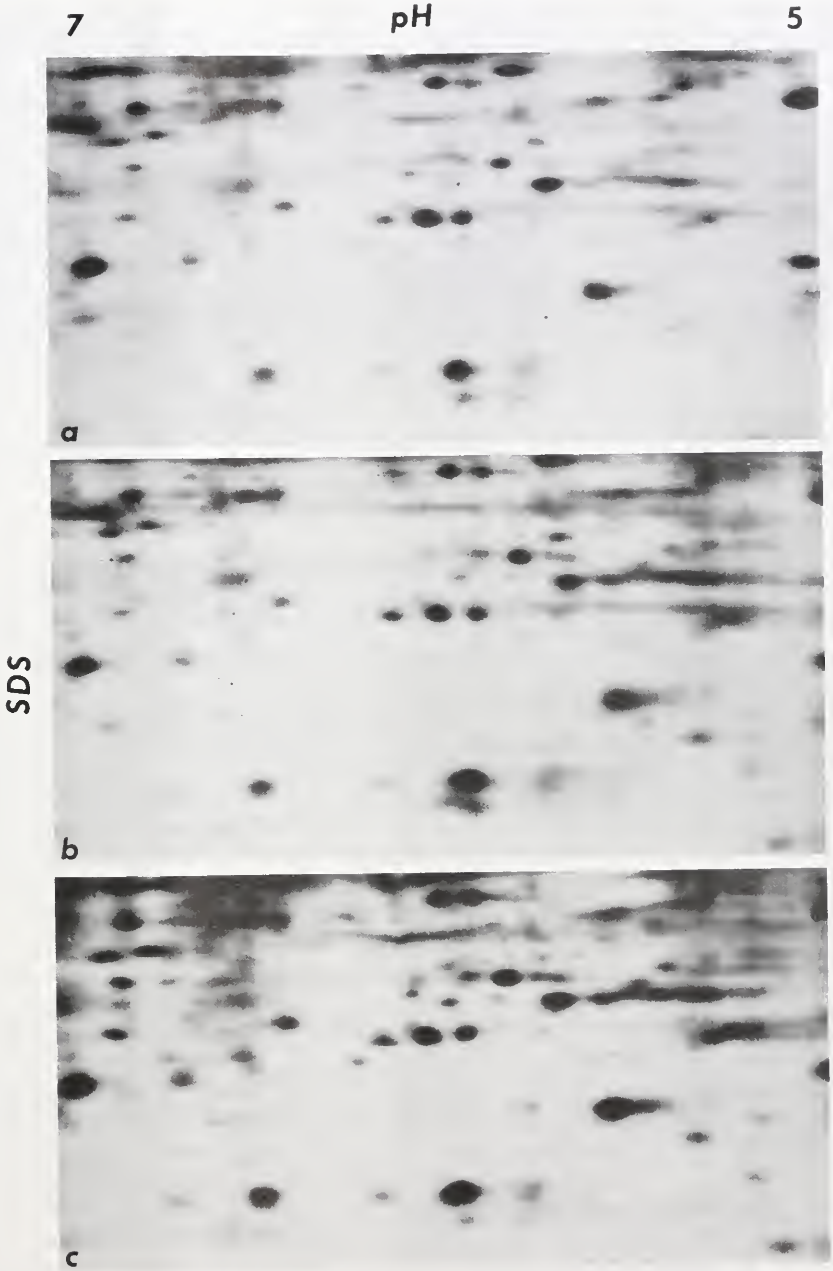


FIGURE 1. Autoradiograph of ^{35}S-methionine *in vivo* labeled polypeptides synthesized during early cleavage by (a) normal embryos, (b) lobeless embryos, and (c) isolated polar lobes.

used in these experiments. These measurements showed that the H4 of the lobeless egg and the isolated polar lobe accumulated 71.2% and 20.7%, respectively, of the amount of methionine incorporated into H4 by the intact egg. This distribution of

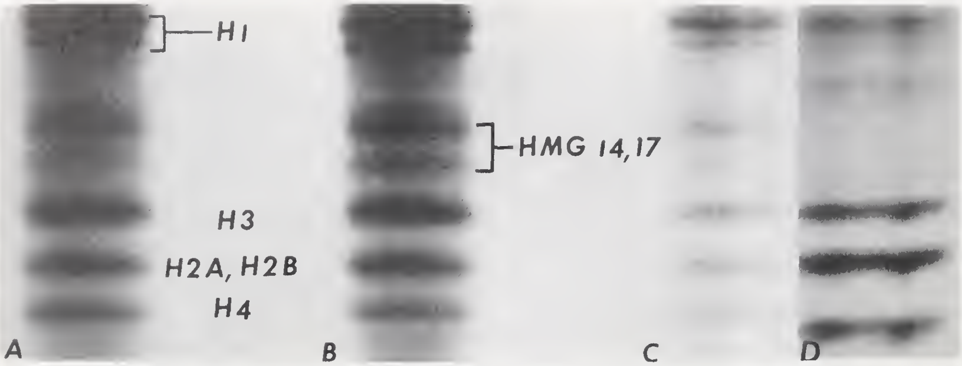


FIGURE 2. Autoradiogrph of *in vivo* labeled histones (H1, H2A, H2B, H3, and H4) and high mobility group peptides 14 and 17 synthesized during early cleavage by (a) normal embryos, (b) lobeless embryos, and (c) isolated polar lobes. In panel (D) are stained histones extracted from isolated nuclei of *Ilyanassa* embryos used, as described in Materials and Methods, to identify the position of histones on the autoradiographs.

label into H4 in these regions of the egg approximates the 84.2% of the volume of non-yolk cytoplasm in the lobeless egg and 16.5% in the polar lobe. Thus, the lobeless egg and the polar lobe probably make histones at the same relative rate, and the histone mRNAs probably are not differentially sequestered into either of these regions of the egg.

Two prominent radioactive bands positioned between the H1 and H3 histones also are resolved in the fluorograph of Figure 2. The location of these bands corresponds to the position of high mobility group peptides 14 and 17 as resolved by SDS acrylamide electrophoresis by Goodwin *et al.* (1979) and Gordon *et al.* (1981). Accordingly, these radioactive bands are probably HMG peptides 14 and 17, and, as shown in these figures, are synthesized not only in the intact egg (Fig. 2A), but also in the lobeless egg (Fig. 2B) and in the isolated polar lobe (Fig. 2C).

DISCUSSION

The nearly complete inhibition of protein synthesis in early-cleavage-stage embryos by cycloheximide demonstrated that mitochondria contribute less than 4% to polypeptide synthesis during cleavage. These results, the observations of Clement and Lehman (1956) that the mitochondria of the *Ilyanassa* egg are concentrated in the animal half of the egg, and the electron micrographs of Pucci-Minafra *et al.* (1969), which also show a paucity of mitochondria in the polar lobe, establish that mitochondria do not contribute significantly to the polypeptide synthesis of the polar lobe or other regions of this egg.

The equivalent rates of protein synthesis measured in different regions of the egg (Table II) could have been an average produced by variable rates of synthesis of different subsets of peptides in the lobeless egg and the polar lobe. However, the similarity in spot size and apparent density of the peptides resolved by two-dimensional electrophoresis (Figs. 1A, B, and C) indicates that all of the detectable proteins made at this stage of development are synthesized at the same relative rate by both the lobeless egg and the isolated polar lobe.

The similar relative rates of protein synthesis in the lobeless egg and the isolated polar lobe indicate that at this stage of development ooplasmic segregation does not differentially localize into the lobeless egg or the polar lobe amino acids (as inferred from the distribution of ^{35}S-methionine) nor rate-limiting components required for protein synthesis (tRNA, Met-tRNA, 40S ribosomal subunits, initiation factors and so on). Therefore, any qualitative differences in polypeptide synthesis in these regions of the egg would not be caused by differences in the amino acid pool nor by competition among mRNAs for rate-limiting components required for protein synthesis. It is possible that the polar lobe does not contain an equivalent complement of these rate-limiting components, but its rate of protein synthesis is equivalent to that of the egg or lobeless egg because the polar lobe contains a higher concentration of mRNA than the egg or lobeless egg. However, this seems unlikely because of the similar concentrations of total RNA in the polar lobe and the whole egg (Collier, 1960), and because polar lobe removal does not alter the proportion of nascent RNA polyadenylated by lobeless eggs during early cleavage (Collier, 1975).

The fluorographs of peptides made by the egg and its isolates (Fig. 1) show that the lobeless egg and the polar lobe contain, within the limits of the peptides detected, the same set of mRNAs and that these mRNAs are not differentially translated in either the animal or vegetal half of the egg. The similarity of the peptides synthesized by the normal egg (Fig. 1A) and the lobeless egg (Fig. 1B) demonstrates that the polar lobe cytoplasm does not regulate, either quantitatively or qualitatively, the proteins whose synthesis was detected during early cleavage. Obviously, these conclusions apply only to the mRNAs whose translation products are labeled by *in vivo* incubation with ^{35}S-methionine and resolved by two-dimensional electrophoresis. The mRNAs that code for the polypeptides detected by two-dimensional electrophoresis probably belong to the super-abundant and moderately prevalent classes of mRNAs (Davidson and Britten, 1979).

Because the same set of peptides synthesized in the normal egg (Fig. 1A) were also made in the isolated polar lobe (Fig. 1C), the availability of the mRNAs coding for these peptides probably did not depend on concurrent gene transcription. Because the chromosomes of the *Ilyanassa* egg were in a nontranscribing stage, *e.g.* meiotic and mitotic divisions, continuously since before fertilization and the time when the polar lobe was isolated, the mRNAs for these proteins probably were transcribed during oogenesis and stored in the egg cytoplasm. This may apply only to the translation of mRNAs contained in the isolated polar lobe; the maternal transcripts of the egg may have been supplemented by mRNAs transcribed from the nuclear genome of the egg during the 4 h incubation with ^{35}S-methionine, *i.e.* from the two- to the twelve-cell stage. However, experiments with eggs continuously incubated in actinomycin D (Collier and McCarthy, in press) ruled out this interpretation by showing that all of these peptides were translated from maternal transcripts and that these maternal mRNAs were not supplemented by embryogenic transcripts during early cleavage.

The present observations on polypeptide synthesis show that the absence of the polar lobe has no discernible effect on protein synthesis during early cleavage, *i.e.* from the two- to the twelve-cell stage. Accordingly, within the limits of polypeptide detection, the expression of structural genes is not associated with the lobe-dependent determinative events of early cleavage. The absence of an immediate effect is not surprising in the light of Clement's (1962) findings that some of the principal morphogenetic influences of the polar lobe are mediated after the 12-cell stage of development.

The similar rates of synthesis of histones in the egg, lobeless egg, and isolated polar lobe shows that the histone mRNAs are not uniquely localized in the animal or vegetal half of the *Ilyanassa* egg, and are translated with equal effectiveness in both regions of the egg. Since the mRNAs for the histones and the HMG peptides 14 and 17 are translated in the isolated polar lobe, their presence does not depend on concurrent gene transcription. Because the chromosomes of the *Ilyanassa* egg were, as discussed above, in a non-transcribing stage before isolation of the polar lobe, the mRNAs for these groups of proteins probably were transcribed during oogenesis. It has not been established that synthesis of these basic proteins is insensitive to actinomycin D. Therefore, the maternal transcripts of histone and HMG peptides may have been supplemented by embryogenic transcripts of histone and HMG peptides produced from the two- to the twelve-cell stage, as, for example, has been demonstrated during early sea urchin embryogenesis (Ruderman and Gross, 1974; Easton and Whitely, 1979).

ACKNOWLEDGMENTS

I thank Miss Arlene Hughes and Miss Mona Friedman for their excellent technical assistance. This research was supported by grants 11624 and 12276 from the City University of New York PSC-BHE Research Award Program and by grant PCM78-02773 from the National Science Foundation.

LITERATURE CITED

BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1:** 279–285.

CLEMENT, A. C. 1952. Experimental studies on germinal localization in *Ilyanassa.* I. The role of the polar lobe in determination of the cleavage pattern and its influence in later development. *J. Exp. Zool.* **121:** 593–626.

CLEMENT, A. C. 1956. Experimental studies on germinal localization in *Ilyanassa.* II. The development of isolated blastomeres. *J. Exp. Zool.* **132:** 427–446.

CLEMENT, A. C. 1962. Development of *Ilyanassa* following removal of the D macromere at successive cleavage stages. *J. Exp. Zool.* **149:** 193–216.

CLEMENT, A. C., AND F. E. LEHMAN. 1956. Uber das Verteilungsmuster von Mitochondrien un Lipoidtropfen wahrend der Furchung des Eies von *Ilyanassa obsoleta* (Mollusca, Prosobranchia). *Die Naturwiss.* **20:**478–479.

CLEMENT, A. C., AND A. TYLER. 1967. Protein-synthesizing activity of the anucleate polar lobe of the mud snail *Ilyanassa obsoleta. Science* **158:** 1457–1458.

COLLIER, J. R. 1957. A study of alanylglycine dipeptidase activity during the development of *Ilyanassa obsoleta. Embryologia* **3:** 1–17.

COLLIER, J. R. 1960. The localization of ribonucleic acid in the egg of *Ilyanassa obsoleta. Exp. Cell Res.* **21:** 126–136.

COLLIER, J. R. 1975. Polyadenylation of nascent RNA during the embryogenesis of *Ilyanassa obsoleta. Exp. Cell Res.* **95:** 263–268.

COLLIER, J. R., AND M. E. MCCARTHY. 1979. Protein synthesis in the polar lobe of the *Ilyanassa* egg. *Amer. Zool.* **19:** 950.

COLLIER, J. R., AND M. E. MCCARTHY. In press. Regulation of polypeptide synthesis during early embryogenesis of *Ilyanassa obsoleta. Differentiation* **19.**

CRAMPTON, H. E. 1896. Experimental studies on gasterpod development. *Wilhelm Roux Arch. Entwicklungsmech.* **3:** 1–19.

DAVIDSON, E. H., AND R. J. BRITTEN. 1979. Regulation of gene expression: possible role of repetitive sequences. *Science* **204:** 1052–1059.

EASTON, D. P., AND A. H. WHITELEY. 1979. The relative contribution of newly synthesized and stored messages to H1 histone synthesis in interspecies hybrid echinoid embryos. *Differentiation* **12:** 127–133.

GARRELLS, J. I. 1979. Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. *J. Biol. Chem.* **254:** 7961–7977.

GOODWIN, G. H., C. G. P. MATHEW, C. A. WRIGHT, C. D. VENKO, AND E. W. JOHNS. 1979. Analysis of the high mobility group proteins associated with salt-soluble nucleosomes. *Nucleic Acid Res.* **7:** 1815–1835.

Gordon, J. S., J. Bruno, and J. J. Lucas. 1981. Heterogeneous binding of high mobility group chromosomal proteins to nuclei. *J. Cell Biol.* **88:** 373–379.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

McCarthy, M. E., and J. R. Collier. 1980. Regulation of polypeptide synthesis in the *Ilyanassa* embryo. *J. Cell Biol.* **83:** 418.

Newrock, K., and B. Brandhorst. 1979. Selective translation of cytoplasmic messenger RNA during embryonic development. *J. Cell. Biol.* **83:** 418.

O'Farrel, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250:** 4007–4021.

Pucci-Minafra, S. Minafra, and J. R. Collier. 1969. Distribution of ribosomes in the egg of *Ilyanassa obsoleta. Exp. Cell Res.* **57:** 167–178.

Ruderman, J. V., and P. R. Gross. 1974. Histones and histone synthesis in sea urchin development. *Dev. Biol.* **36:** 286–298.

Schatz, G., and T. L. Mason. 1974. The biosynthesis of mitochondria proteins. *Ann. Rev. Biochem.* **43:** 51–88.

Wilson, E. B. 1928. *The cell in development and heredity.* Macmillan Co., New York. 1231 pp.

Reference: *Biol. Bull.* **160:** 376–382. (June, 1981)

FISH MUCUS: *IN SITU* MEASUREMENTS OF POLYMER DRAG REDUCTION

THOMAS L. DANIEL

*Department of Zoology, University of Wisconsin, Madison, Wisconsin 53706**

ABSTRACT

The external layer of mucus on fish was investigated as a drag reducing polymer. Comparing velocity profiles for water flow over rainbow trout (*Salmo gairdneri*) and wax models of trout with and without hydrodynamically smooth surfaces revealed that the integumental mucous secretion can significantly reduce the rate of momentum transfer through the boundary layer. The difference in momentum transfer is expressed as a reduction in friction drag and discussed in view of the overall drag experienced by fish.

INTRODUCTION

Virtually all fishes are covered with an integumental mucous secretion that is involved in many aspects of their biology, ranging from disease resistance and rearing of young to shelter and locomotion (Jakowska, 1963; Winn, 1955). In locomotion, the layer of mucus is assumed to reduce friction drag. Dissolved in water, these external secretions have been shown to reduce friction drag in turbulent pipe flow (Hoyt, 1975; Ling and Ling, 1974; Rosen and Cornford, 1971) and pulsed laminar flow (Driels and Ayyash, 1976). These results have been attributed to the "Toms effect" (after Toms, 1948), in which dilute solutions of high-molecular-weight polymers flowing in tubes yield friction-drag coefficients less than values predicted for Newtonian fluids. Most authors agree that fish mucus reduces drag. No one, however, has estimated the degree to which mucus can decrease the friction drag experienced by swimming fish.

The drag-reducing ability of fish mucus in solution has been determined by a turbulent flow rheometer (Hoyt, 1965, 1975; Rosen and Cornford, 1971). In addition, Ling and Ling (1974) investigated the velocity field in the laminar sublayer of mucus injected into water flowing in a tube. In all these experiments, mucus samples were removed from fish and analyzed for drag reduction as a function of mucus concentration. In reality, mucus is secreted by the integument and diffuses into water at some unknown rate. The concentration gradients are unknown. Hence, the drag reduction is also unknown.

In the present paper, the drag-reducing ability of mucus is investigated by comparing velocity profiles of the flow over the surface of freshly killed fish to those over wax models with and without smooth surfaces. Direct measurements of the velocity profiles provide estimates of the rates at which momentum is transferred from the free-stream flow (a region in which the effects of a boundary are trivial) to the surface of the fish or wax models. These profiles are used, in turn, to determine

Received 19 January 1981; accepted 2 March 1981.

Abbreviations: f_x, local friction factor; Re_x, local Reynolds number; v_x, local fluid velocity in cm/s v, free-stream fluid velocity in cm/s; y, height above surface of fish in cm; μ, dynamic viscosity of water ($\times 10^{-2}$) in $g \cdot cm^{-1} \cdot s^{-1}$; ρ, density of water in g/cm^3; τ_0, shear stress at surface in $g/(cm \cdot s^2)$.

* Present address: Department of Zoology, Duke University, Durham, North Carolina 27706.

the reduction in friction drag resulting from mucus in the boundary layer (a region of flow between the free-stream and the surface of the fish, where the effects of viscosity are quite strong).

METHODS

Density and viscosity measurements

Mucus of healthy rainbow trout (*Salmo gairdneri*, Richardson) was removed by rubbing the fish from head to tail with sterile gloved hands. The mucus was transferred from the tail to a sterile petri dish and used within 1 h. Its density was measured over a temperature range of 10–31°C by standard pycnometric technique using a 2.0-ml pycnometer.

A Ferranti-Shirley cone and plate viscometer was used to measure mucus viscosity in shear rates less than 8000 s^{-1}. Mucus samples for viscometry were collected as above.

Flow visualization

Use of an open-circuit flow tank avoided the possibility of recycling polymers shed by the fish (Fig. 1). Flow through the 14.0-cm-diameter test chamber was controlled by a downstream tube of flexible polyethylene equipped with a C-clamp.

A 35-mm camera and a Strobotac stroboscope were placed on opposite sides of the test chamber. A 50-mm lens with extension tubes provided a shallow depth of field and a 1.7-fold magnification on the film.

Two wax models were made from a plaster cast of a 19.0-cm-long trout. One was coated with silicone lubricant (stopcock grease) to simulate the hydrodynamic smoothness of a layer of mucus. The other model was left untreated, retaining the surface features of the scales.

Fish 19–20 cm long were killed by a sharp blow to the skull and placed on a platform in the test chamber with their lateral sides up and dorsal sides facing the camera. The camera was focused on a portion of the lateral side of the fish 8 cm

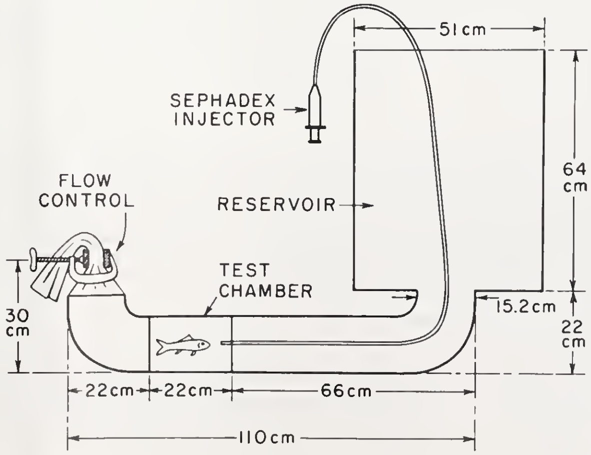


FIGURE 1. Schematic diagram of the flow system used in the study. The reservoir capacity is 120 l. The test chamber is a Plexiglas tube with an inside diameter of 14 cm. The Sephadex injector consists of a 60 cm^3 syringe and 200 cm of 0.2 cm diameter Tygon tubing.

from the snout. The flow tank exit was then opened and G-25 coarse-grained Sephadex particles (100–300 μm diameter) were injected 2–4 cm in front of the fish. Photographs were taken at a shutter speed of 0.25 s with a stroboscope frequency of 32 flashes/s, giving eight images of each particle on a single frame. The procedure was repeated for the wax models with and without silicone lubricant.

Velocity profiles

The distances that Sephadex particles traveled between strobe flashes and their heights above the surface of the fish were measured to within 40 μm with a caliper and a microscope, from negatives. Two intersecting line segments were fitted to the data by a least-squares linear regression (Hudson, 1966). Thus, each resulting velocity profile consisted of two lines: an upper line corresponding to the free-stream velocity and a lower segment corresponding to the boundary layer flow. The lower segment was operationally defined as the velocity gradient in the boundary layer ($\partial v_x/\partial y$, where v_x is fluid velocity in cm/s, and y is cm height above the fish's surface). Thus, a series of such profiles could be used to obtain data for the boundary layer velocity gradient as a function of free-stream velocity. This gradient, in turn, was used to describe momentum transfer and friction drag on the fish and wax models.

RESULTS

Density and viscosity measurements

In no case did the density of mucus differ from that of water by more than 1%. Cone and plate viscosimetry indicated that the viscosity of mucus was about the same as water; that is, about 1 centipoise. However, the viscosity of a polymeric solution such as mucus is sensitive to degradation of high-molecular-weight fractions (White and Hemmings, 1976). I have no estimate of the degree of degradation that might have occurred between the time the samples were collected and tested.

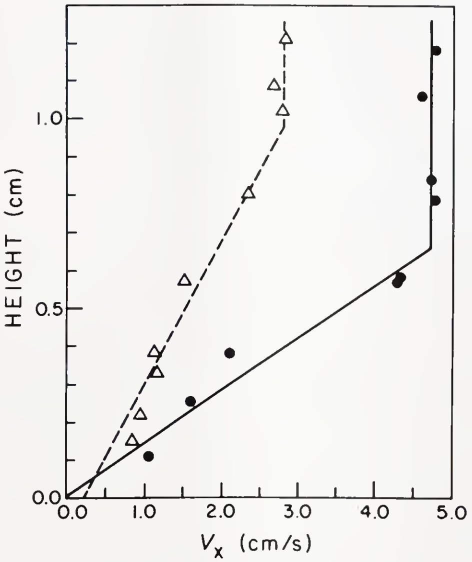
Velocity profiles

Velocity profiles over the surface of a mucus-covered fish are shown in Figure 2, with intersecting line segments fitted to the data for two free-stream velocities. As the free-stream velocity increased, the velocity gradient also increased. The velocity gradients were 2.6 s^{-1} for the low flow rate and 7.2 s^{-1} for the high flow rate.

Figure 3 compares the boundary-layer velocity profile over the surface of a fish to that over the smooth wax model. The velocity gradient for the fish was approximately 40% lower. Since the rate of momentum transfer from the free-stream flow to the surface of the fish is proportional to the velocity gradient, the results summarized in Figure 3 suggest that mucus on the surface of the fish decreased momentum transfer and, ultimately, friction drag.

Double-segment regressions like those in Figure 2 were used to determine the degree to which mucus reduced the rate of momentum transfer to the surface of the fish. The slope of the lower segment represents the average velocity gradient within the boundary layer. This slope was used to approximate the shear stress acting on the surface of the fish and models using Newton's law of viscosity.

$$\tau_0 = -\mu \frac{\partial v_x}{\partial y}$$



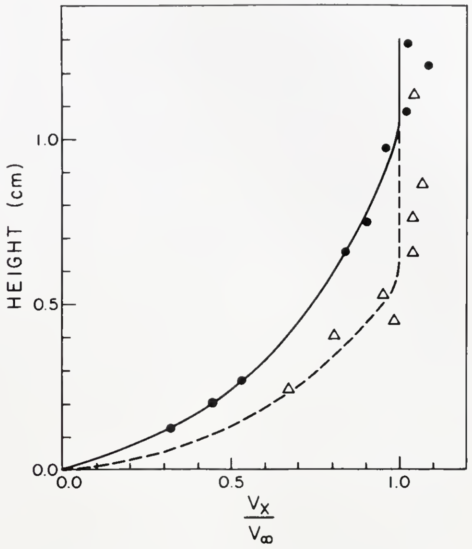


FIGURE 2. Height above the surface of the fish plotted against velocity (v_x) to give a velocity profile. Solid circles and triangles correspond to a free-stream velocity of 4.7 and 2.8 cm/s respectively. The slanted portion of the curves represents the velocity gradient in the boundary layer. The regression for the left segment of the solid line is $v_x = 0.05 + 7.17$ (height) ($r^2 = 0.932$, $N = 5$) and for the broken line is $v_x = 0.24 + 2.6$ (height) ($r^2 = 0.955$, $N = 6$).

FIGURE 3. Height above the surface is plotted against normalized velocity (v_x/v) for the fish (solid circles) and wax model with silicone lubricant (triangles). The free-stream velocities about the fish and model are, respectively, 2.15 and 2.19 cm/s.

where τ_0 is the shear stress at the surface of the fish, μ is the dynamic viscosity of water, and v_x is the local velocity of water at any height (y) above the surface.

The local friction factor was defined as the ratio of wall shear stress to kinetic energy per unit volume of fluid in the free stream by the following relationship (DeNevers, 1970):

$$f_x = \tau_0(½\rho v_\infty^2)^{-1}$$

where f_x is the local friction factor, ρ is the density of water and v_∞ is the free-stream velocity.

In Figure 4, the local friction factor is plotted against the local Reynolds number for a variety of flows over the fish and wax models. The log of the local friction factor versus the log of the local Reynolds number was fitted by least-squares linear regression. The regression lines for the wax models with and without a smooth surface were not significantly different. The regression line for the fish was significantly lower than those from the wax models (*F* test, $P < 0.025$). The slope of the line for friction-factor versus Reynolds number was more negative for the fish, although not significantly different from the wax models. The solid line in Figure 4 is the predicted local friction factor for flow over a flat plate in laminar flow (DeNevers, 1970). The local friction factor for the wax models approximates the local friction factor for a flat plate at the same Reynolds number. This is in accordance with expected results for a streamlined body (Goldstein, 1965).

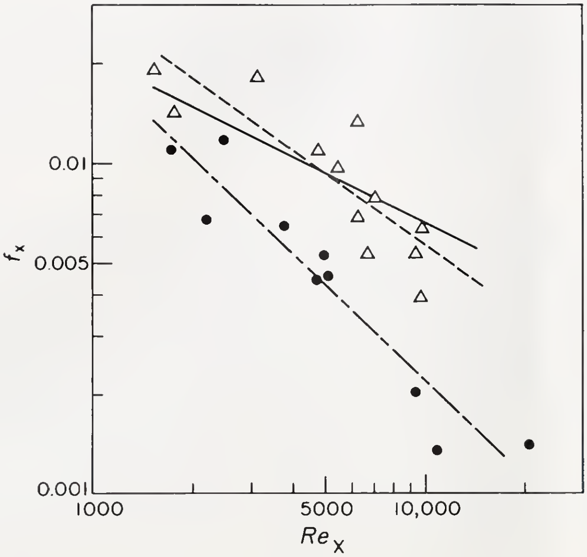


FIGURE 4. Local friction factor (f_x) is plotted against the local Reynolds number (Re_x) for the fish (solid circles) and both wax models (triangles). The solid line corresponds to the predicted friction factor for flow over a flat plate in laminar flow. The broken lines represent the regression equations which for the fish is $f_x = 14.45\ Re_x^{-0.95}$ ($r^2 = 0.88$, N = 11) and for the wax models is $f_x = 4.69\ Re_x^{-0.73}$ ($r^2 = 0.70$, N = 12). The slopes of these lines are not significantly different.

DISCUSSION

The velocity gradient about an object moving through a fluid can be considered a measure of the transfer of momentum from fluid in the free-stream to the surface of the object, by the action of viscosity: the greater the velocity gradient, the greater the rate of momentum transfer. The velocity gradient can be modified by changes in surface texture (Goldstein, 1965) and, as this study has shown, by mucus in the boundary layer. The velocity gradient about a fish is less steep than that about a hydrodynamically smooth model of the animal. This difference reduces the local friction factor on the order of 50%, with a trend towards a greater reduction at higher Reynolds numbers. The reduction in frictional drag may reasonably be attributed to mucus. The possibility that mucus on fish is behaving as a "Toms effect" solution should be considered. In this case, high molecular weight polymers from the mucus may be decreasing the rate of momentum transfer from the fluid to the animal's surface.

Several conditions must be satisfied for mucus to act as a drag reducer and for my treatment of velocity gradients to be valid: (1) Mucus must consist, in part, of polymers soluble in water and with molecular weights exceeding 50,000 (White and Hemmings, 1976). (2) There must be turbulent or pulsed laminar flow about the fish (Driels and Ayyash, 1976). And (3) the density and viscosity of the fluid from the surface of the fish outwards must be constant.

Existence of the first condition is supported by studies of mucus/water solutions subjected to turbulent flow in pipes (Hoyt, 1975; Rosen and Cornford, 1971). Regarding condition (2), however, I am not aware of any analyses of the size distribution of polymers in fish mucus. The velocity profiles presented here indicate that the flow about the fish and wax models is turbulent, supporting the second condition. Although the local Reynolds number for flow over the fish does not indicate turbulence, the Reynolds number for flow in the tank exceeded 2000 in all situations, indicating a turbulent flow regimen was imposed on the fish and wax models. Swimming fish most likely experience turbulent flow or, with body undulations, pulsed laminar flow. Lastly, constant density and viscosity within the boundary layer is supported by the present measurements.

This study shows that mucus decreases the friction drag experienced by fish. The total drag on a swimming fish consists of pressure drag as well as friction drag. Webb (1978) estimated total drag coefficients of swimming fish, using measured power outputs. The relative importance of friction drag to total drag, however, remains unknown. Estimates of total drag using rigid models or dead fish (*e.g.* Gero, 1952) are difficult to apply to a moving animal due to the problems of (1) analyzing the pressure field about an undulating body (Wu, 1977), (2) the unknown nature of mucus, or lack thereof, on a dead fish or rigid model, and (3) the unknown flexibility of models or fish. While the present study suggests that the rainbow trout has a mechanism by which it decreases friction drag, such a reduction may not decrease total drag. The contribution of friction drag to total drag is, at present, unknown, and may be a small portion of total drag. Wu (1977), however, suggests that for a streamlined body such as a fish, the majority of drag is frictional. Lastly, there is evidence that polymer additives can delay the point of wake separation, reducing the size of the wake and the pressure drag; and that this effect can be seen in laminar flows (White and Hemmings, 1976).

Polymer drag reduction is by no means limited to teleosts, and may not be limited to the individual producing mucus. Breder (1976) suggests that schooling fish may take advantage of polymer drag reduction. He observed that fish at the trailing end of a school beat their tails less often than did fish at the leading edge, indicating a reduction in drag arising from dissolved polymers from the leading fish. Mucus secretion and its role in drag reduction can be found in a variety of biological situations. Hoyt (1970, 1974) has shown that mucus from marine organisms such as algae and bacteria can reduce drag. Moreover, many marine animals, such as cephalopods, coelenterates, and sponges, have mucus on their surfaces. The study of forms where separate components of drag can be determined would provide much insight into the role of mucus in drag reduction.

ACKNOWLEDGMENTS

I wish to thank M. Wrabetz for help in collecting data. W. Porter, S. Vogel, and the members of the University of Wisconsin Biophysical Ecology Laboratory provided many useful suggestions. I am grateful to two anonymous reviewers for their criticisms of the manuscript. Computer time was provided by DOE grant EY-76-02-2270 to W. Porter.

LITERATURE CITED

BREEDER, C. M. 1976. Fish schools as operational structures. *Fish. Bull.* **76:** 471–502.

DENEVERS, N. 1970. *Fluid mechanics.* Addison-Wesley, London. 508 pp.

DRIELS, M. R., AND S. AYYASH. 1976. Drag reduction in laminar flow. *Nature* **259:** 389–390.

GERO, D. R. 1952. The hydrodynamic aspects of fish propulsion. *Am. Mus. Novit.* **1601:** 1–32.

GOLDSTEIN, S. 1965. *Modern developments in fluid mechanics, Vol. 2.* Dover Publications, New York. 702 pp.

HOYT, J. W. 1965. A turbulent flow rheometer. *Am. Soc. Mech. Eng., App. Mech. and Fluid Eng. Conf.*, pp. 71–82.

HOYT, J. W. 1970. High molecular weight algal substances in the sea. *Mar. Biol.* **7:** 93–99.

HOYT, J. W. 1974. Recent progress in polymer drag reduction. *Colloq. Int. Cent. Natl. Rech. Sci.* **223:** 193–215.

HOYT, J. W. 1975. Hydrodynamic drag reduction due to fish slimes. Pp. 653–673 in T. V. T. Wu, C. J. Brokaw, and C. Brennon, Eds., *Swimming and flying in nature.* Plenum Press, New York.

HUDSON, D. J . 1966. Fitting segmented curves whose join points have to be estimated. *Amer. Stat. Assoc. J.* **6:** 1097–1129.

JAKOWSKA, S. 1963. Mucus secretion in fish—a note. *Ann. N. Y. Acad. Sci.* **160:** 458–462.

LING, S. C., AND T. Y. LING. 1974. Anamolous drag-reducing phenomenon at a water/fish-mucus or polymer interface. *J. Fluid Mech.* **65:** 499–512.

ROSEN, M. W., AND N. E. CORNFORD. 1971. Fluid friction of fish slimes. *Nature* **234:** 49–51.

TOMS, B. A. 1948. Some observations on the flow of linear polymer solutions through straight tubes at large Reynolds numbers. *Proc. Internat. Rheol. Cong., Holland* **2:** 135–141.

WEBB, P. W. 1978. Hydrodynamics: nonscombroidfish. Pp. 189–238 in W. S. Hoar and D. J. Randall, Eds., *Fish physiology, Vol. V.* Academic Press, New York.

WHITE, A., AND J. A. G. HEMMINGS. 1976. *Drag reduction by additives: review and bibliography.* BHRA Fluid Engineering, Bedford, England. 181 pp.

WINN, H. E. 1955. Formation of a mucous envelope at night by parrot fishes. *Zoologica* **40:** 145–148.

WU, T. Y. 1977. Introduction to the scaling of aquatic animal locomotion. Pp. 203–232 in T. V. T. Wu, C. J. Brokaw, and C. Brennon, Eds., *Swimming and flying in nature.* Plenum Press, New York.

Reference: *Biol. Bull.* **160**: 383–393. (June, 1981)

MORPHOLOGY OF LEECH SENSILLA: OBSERVATIONS WITH THE SCANNING ELECTRON MICROSCOPE

Y. SUSAN DEROSA AND W. OTTO FRIESEN

Department of Biology, University of Virginia, Charlottesville, Va 22901

ABSTRACT

The external morphology of the body wall of the medicinal leech, *Hirudo medicinalis*, was investigated with the scanning electron microscope. At high magnification, most of the leech body wall has a granular appearance punctuated by numerous small pores. The disc-shaped sensory sensilla are easily distinguished from the surrounding body wall by the absence of pores and the presence of numerous filiform projections in the central region of each sensillum. Two types of projections can be distinguished: single, 3–9 μm long "S-hairs" and grouped (*i.e.*, composed of several subunits), 1–2 μm long "G-hairs." Each sensillum supports 40–90 S-hairs and 15–20 G-hairs. The S-hairs may be the sensory structures mediating leech sensitivity to low amplitude water movements.

INTRODUCTION

Several types of sensory receptors occur in the leech body wall. One type, free nerve endings in the epidermal layer, has recently been shown to be the sensory terminals of touch-sensitive neurons (Blackshaw and Nicholls, 1979). Other cutaneous receptive endings must mediate sensitivity to pressure and to noxious stimuli (Nicholls and Baylor, 1968). In addition to these generalized tactile mechanoreceptors, leeches have localized receptors: eyes and segmental sensilla. The eyes, photosensory structures at the anterior end of the animal, may have sufficient complexity to provide some rudimentary vision of forms (Mann, 1961). In the medicinal leech, the segmental sensilla are 14 disc-shaped sensory structures on the middle one of five annuli that delineate each segment of the midbody region. Sensilla are also found in the head and tail regions.

It has long been thought that the segmental sensilla, as well, mediate light sensitivity (Whitman, 1886). This was demonstrated electrophysiologically by Kretz *et al.* (1976). As in leech eyes, the photoreceptors evidently consist of a few spherical refractile cells whose axons project centrally via the sensillar nerve (Whitman, 1886). In addition to these photoreceptors, the sensilla contain numerous elongated sensory cells, some of which appear to have filiform processes extending beyond the overlying cuticle (Mann, 1961). The function of these elongated sensory cells remains unknown, although Herter (1929) suggested that the sensilla may mediate sensitivity to water movements. Mann (1961) suggested that the sensilla also may mediate chemosensitivity.

Recent physiological experiments with medicinal leeches showed that the sensilla are indeed the locus for the sensory receptors mediating the sensitivity to water movements (Friesen, 1981). In this paper, we report the results of an investigation with the scanning electron microscope of the external morphology of segmental sensilla in the leech, *Hirudo medicinalis*. We found that midbody sensilla are

Received 18 February 1981; accepted 26 March 1981.

composed of two morphologically distinct regions: (1) a central disc bearing two types of filiform processes, surrounded by (2) a pore-free, apparently raised ring not bearing any filiform processes. Leech sensitivity to water movements could be mediated by one of the two types of filiform processes.

MATERIALS AND METHODS

Hirudo medicinalis specimens obtained from a commercial supplier were kept in aquaria at 20°C. The 20 leeches used for these studies were 7–10 cm long and had not been fed recently. Dissections were performed on leeches anesthetized by cold Ringer's solution (Friesen, 1981) containing 8% ethanol.

Two tissue preparations were used: whole leeches and body-wall sections composed of three or four segments. Mucus was removed from the tissue by brief rinsing in 8% ethanol. The tissue was then fixed with 2% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.5). During fixation, the sensilla were located by examining the body wall sections under the dissecting microscope. The tissue was then nicked at two edges so that the intersection of lines from the nicks would coincide with the position of the sensilla. This made it easier to find the sensilla when viewed with the scanning electron microscope (SEM) and made it possible to compare the structures seen with the light microscope with those observed with the SEM.

Following fixation, the tissues were rinsed in 3.5% sucrose in 0.1 *M* phosphate buffer. Following dehydration with ethanol, the whole leeches were cut into sections containing three or four body segments. After dehydration with a critical point dryer, the tissues were affixed to stubs with Pelca silver paste and coated with a 150–300-Å-thick layer of gold-paladium in a Technic Hummer I. The prepared tissue was examined with an ETEC Model II scanning electron microscope.

To assess shrinkage due to tissue preparation, the dimensions of annuli and sensilla were measured *in vivo*, with the leech in cold Ringer's solution, after fix-

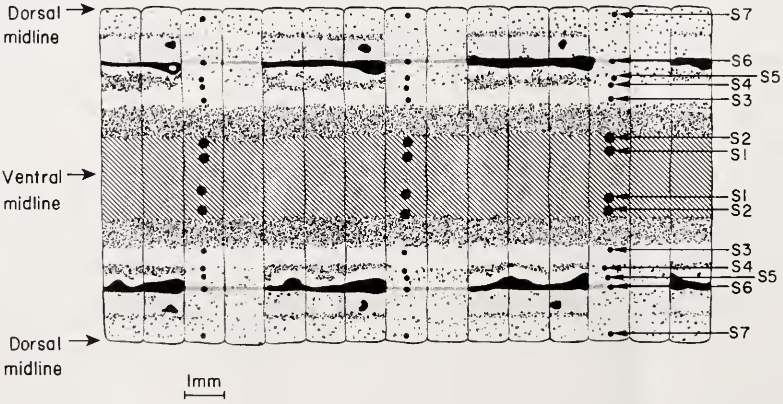
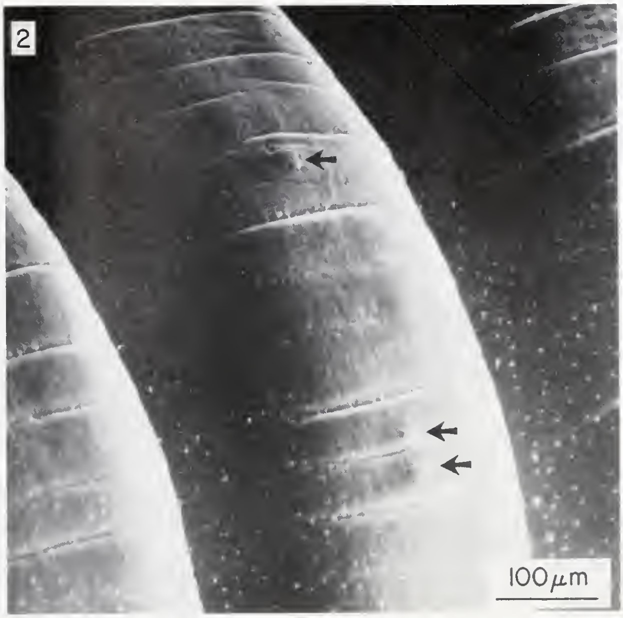


FIGURE 1. The medicinal leech body wall, drawn as viewed with the light microscope. The body wall was slit along the mid-dorsal line and three segments were pinned out flat, with the anterior to the left. Note that each body segment is subdivided into five annuli. The 14 sensilla are located on the middle annulus of each segment at the positions and with the relative sizes shown by the black dots. The stripes at the ventral midline represent yellow, small dots represent orange, and irregular black flecks and solid black represent black. The unmarked background color is green.

2



3

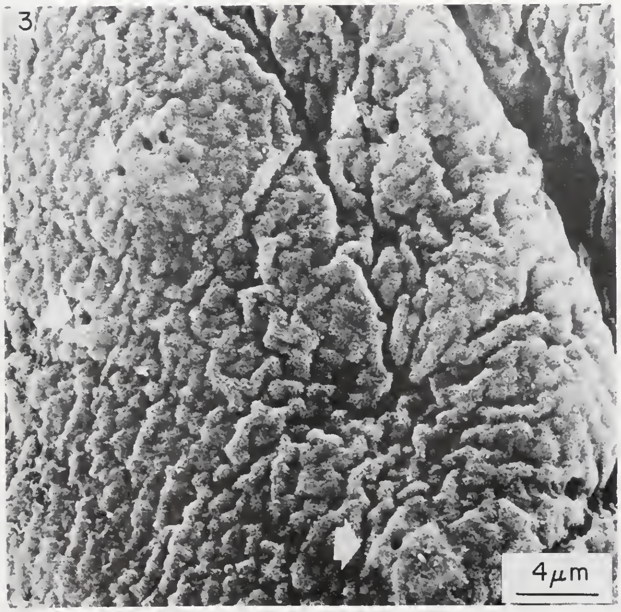


FIGURE 2. Low power SEM photomicrograph of the leech mid-body. The middle annulus of a midbody segment crosses the middle of the figure. The body wall is oriented so that the ventral midline is above the viewed area and anterior is to the left. The arrows indicate structures that occur at every fifth annulus.

FIGURE 3. High power SEM photomicrograph of typical body wall. Deep fissures and shallow grooves may result from muscular contractions. Between grooves, the body wall is characterized by shallow ripples and numerous pores (at arrows).

4

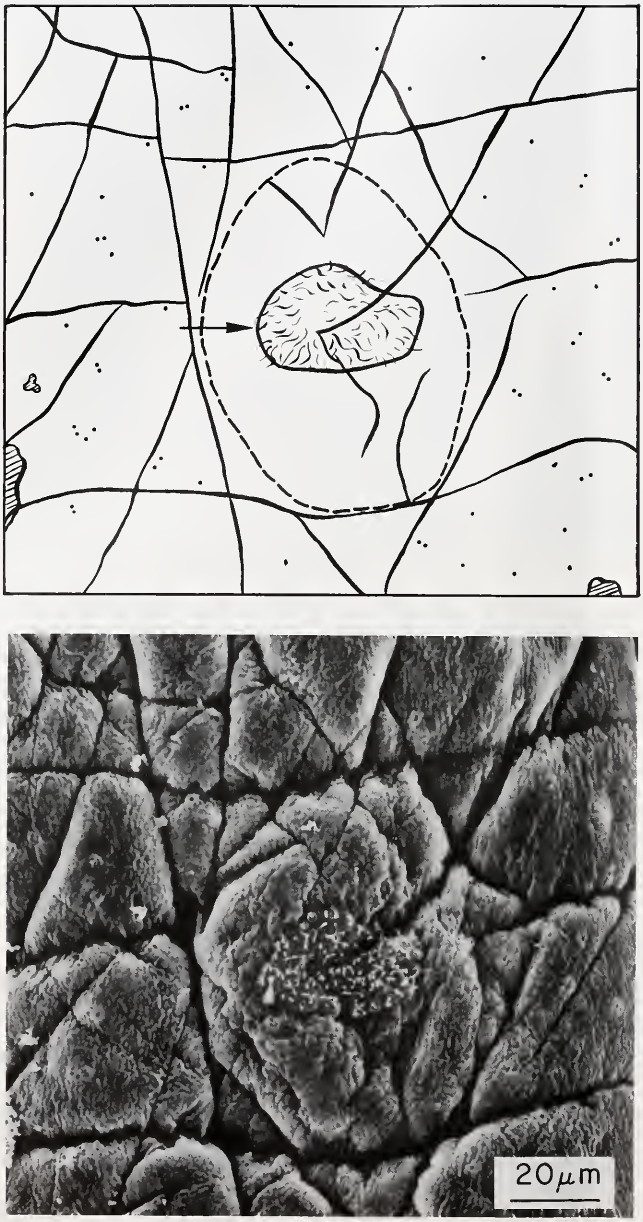


FIGURE 4. Appearance of the leech body wall at sensillum S1. The central area with numerous white projections is surrounded by a 20-μm-wide ring, which like the central area is devoid of pores. The sketch illustrates the extent of the two areas more clearly—the dashed lines indicate the approximate limit of the sensillum.

ation, while in the sucrose solution, and finally after critical point drying. All measurements were made with a dissecting microscope equipped with a reticule. One problem in evaluating dimensions in the leech is that the degree of contraction/

5

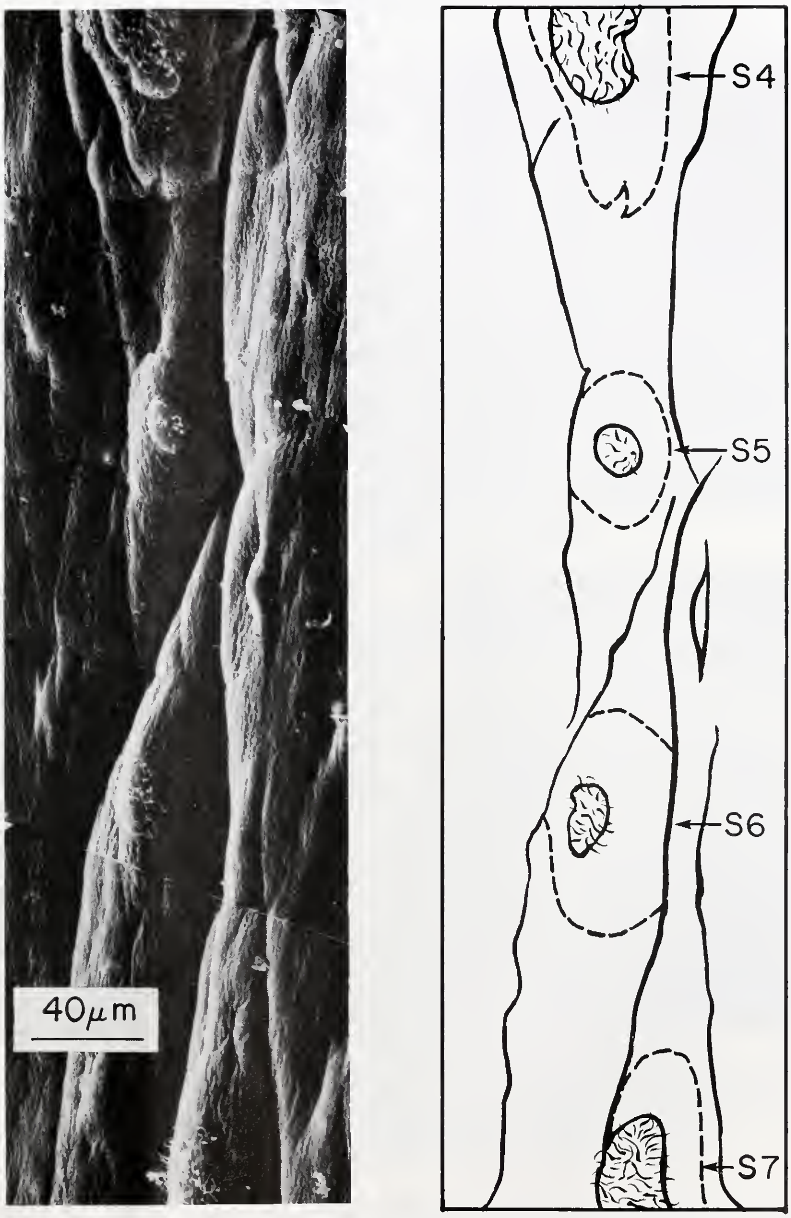


FIGURE 5. Photomicrograph of the middle annulus of the anterior midbody, showing four sensilla: S4, S5, S6, and S7. Both the central region and the surrounding raised ring of each sensillum are evident. The sketch at the right shows the extent of each structure.

relaxation varies not only from animal to animal but also along the individual leech itself. Waves of contraction still passed along the bodies of animals in cold Ringer's solution or in fixative, even though the animals were stretched out. To standardize

6

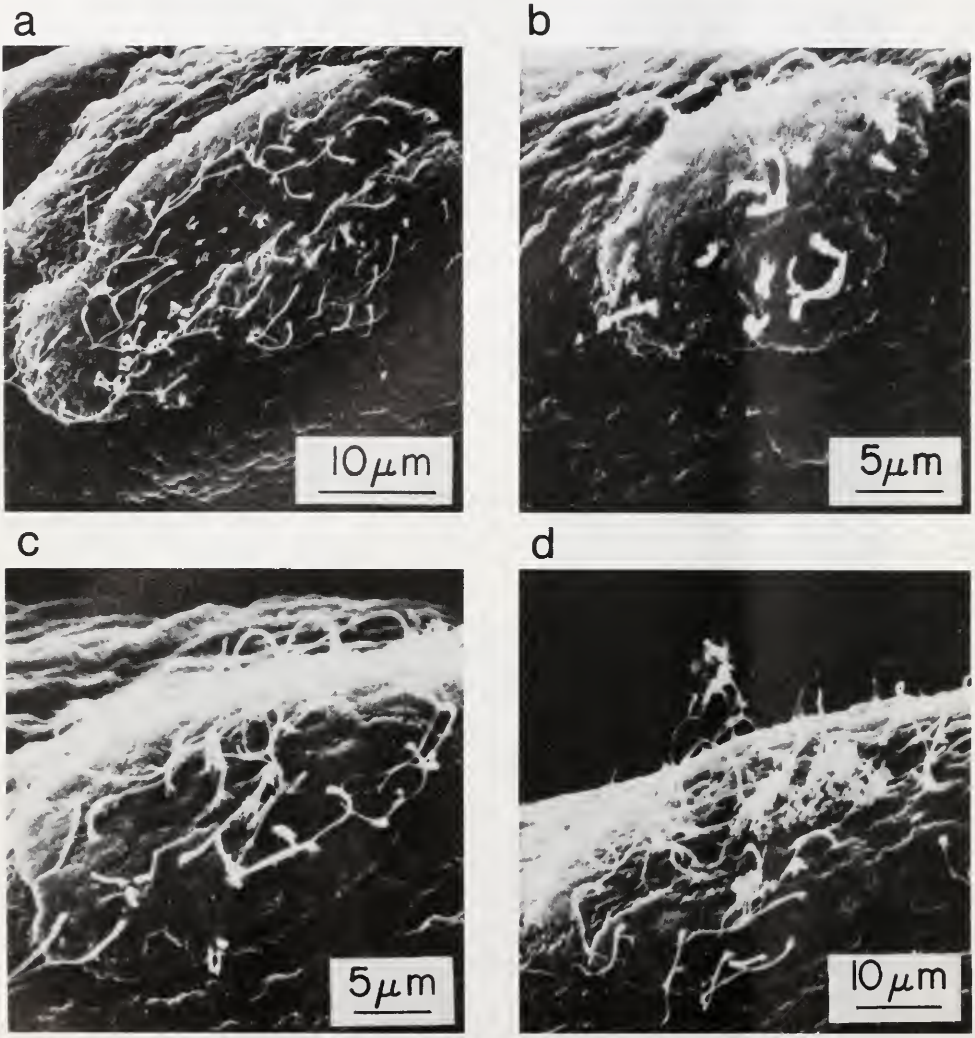
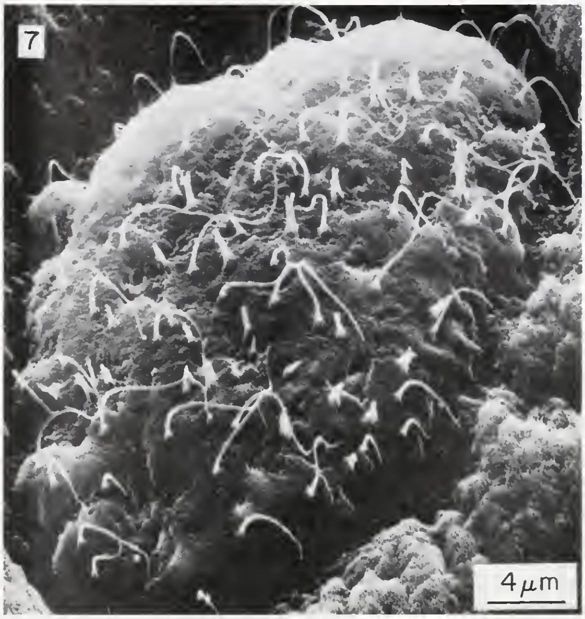


FIGURE 6. The sensilla of Figure 5 at greater magnification. The correspondence between these photomicrographs and the sensilla of Figure 5 is as follows: "a" shows S4; "b", S5; "c", S6; and "d", S7. Note that the viewing angles are not necessarily the same as that of Figure 5 and that magnifications differ.

the annular widths, we measured extended and contracted annuli, and averaged the difference between those two measurements for a number of annuli in the anterior, middle, and posterior regions. This average difference for a given region was then added to the measurements of contracted annuli. This procedure converted all annular widths to those of fully extended annuli. Comparing annular measurements for *in vivo*, fixed, and dried specimens showed that width decreased 16–20% during the drying process, comparable to the shrinkage observed by Hayat (1978).

The nomenclature used to describe leech segmentation is that of Kristan *et al.*

7



8

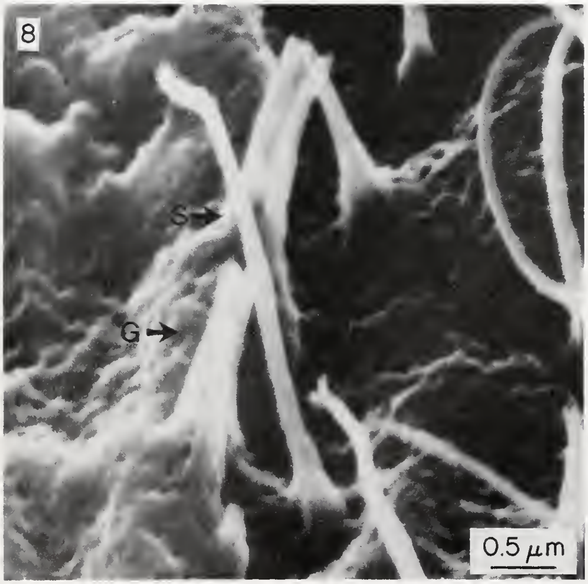


FIGURE 7. Photomicrograph of sensillum S3. Longer single hairs, S-hairs, and shorter grouped hairs, G-hairs, occur. The two hair types are interspersed evenly over the entire central region.

FIGURE 8. High power SEM photomicrograph of the S- and G-hairs located on sensillum S2. The G-hairs occur in tightly apposed groups of two or more and are 0.2–0.4 μm in diameter and 1–2 μm in length. The S-hairs measure 0.2–0.4 μm in diameter and 3–9 μm in length.

(1974). By this numbering scheme, segment 1 is the first of twenty-one midbody segments. We observed only these midbody segments (1 to 21). The sensilla are labeled S1 to S7, according to the scheme of Kretz *et al.* (1976). S1 and S2 are the ventral-most, S3 is positioned laterally, and S4–S7 are on the dorsal aspect of the leech body wall.

RESULTS

Location and dimensions of sensilla

With the dissecting microscope, sensilla can be distinguished from the unspecialized body wall as light grey or semitransparent circular to elliptical areas on the middle annulus of each midbody segment. The actual shape of the sensilla varies depending upon the degree of contraction of the surrounding body wall. In Figure 1, the ventral-most sensilla (S1 and S2) are easily discernable in the light yellow ventral body wall. S1 lies approximately 500 μm from the ventral midline, and S2 about 400 μm more laterally. S3 is in the light yellow band of the lateral edge, sometimes almost within the dark ventrolateral band. S4 and S5 lie in the black and green coloring just dorsal to the lateral yellow band. These two sensilla may be as little as 100 μm apart. The next sensillum, S6, is in the dorsolateral orange band, about one-third of the way from S5 to S7. Finally, S7 is located about 200 μm from the dorsal midline, within the dorsal band. Because of bilateral symmetry, each sensillum has a homonymous counterpart located in the contralateral body wall.

The sensilla fall into two size classes: about 100 μm × 200 μm for the diameters of the minor and major axis of S1 and S2 and about 60 μm × 90 μm for the same dimensions of S3 to S7. Because S1 and S2 are larger and situated in uniformly colored body wall, they are the sensilla most easily discernible with the dissecting microscope.

The leech body wall is very distendable. Sensillar dimensions are therefore only approximate; their deviation from roundness and their apparent size are altered by changes in stretch or contraction of the body wall.

When viewed with the SEM under low magnification, the external body wall appears as a series of rounded ridges—the annuli seen with the unaided eye and the light microscope. Figure 2 shows the lateral aspect of the leech body wall. The rounded band running across the middle of this photomicrograph is the middle annulus of a midbody segment. Parts of adjacent annuli can be seen to either side of this annulus. Numerous crevices in the annuli parallel the anterior-posterior axis of the leech. The small rounded structures (at arrows) mark the location of the sensilla. These structures were observed at the middle annuli of all segments, but not on the intervening annuli.

Identification of the sensilla with the SEM

Examination of the leech body wall with the SEM at high magnification reveals numerous small grooves and fissures (Fig. 3). Between these, the surface is fairly smooth, though still somewhat rippled and generally grainy, possibly due to epidermal projections such as have been described in other leech species (Desser and Weller, 1977). Small pores (arrows, Fig. 3) found over most of the body surface, usually in groups of two or three, probably are the openings of the unicellular epidermal glands observed with the light microscope (Mann, 1961).

Figure 4 is a medium power SEM photomicrograph of a preparation marked to identify its sensilla as those seen with the dissecting microscope. The body wall surface near the perimeter is divided by fissures, appears rippled, and has numerous pores at irregular intervals. However, the center of the picture—at the marked location—is quite different. First, an oval area with white, elongated projections (at arrow in the line drawing) can be seen at the very center. Second, this central area and a surrounding ring are devoid of pores. These two features distinguish the sensilla from the general body wall. This body wall morphology was found in all the marked body wall sections examined. It also was found when the small round structures shown in Figure 2 were examined at high magnification, demonstrating that these structures are, in fact, sensilla.

The composite photomicrograph of Figure 5 shows four sensilla from the middle annulus of one midbody segment at medium magnification. The accompanying sketch illustrates the limits of the sensillar areas. While the body wall pores are not evident at this magnification, the central area of each sensillum is surrounded by a ring of tissue that evidently is slightly elevated from the surrounding body wall.

Sensillar substructure

The question of whether the two morphologically distinct regions at the sensilla both make up the sensillar disc seen with the light microscope was answered by comparing the dimensions of the structures. The average diameter of the central region of the structure seen with the SEM is about 20 μm, much smaller than the sensillar diameters measured with the light microscope, even allowing for shrinkage. Thus, the central sensillar region bearing the white projections is a distinct substructure of the sensilla. The raised, pore-free ring surrounding this central region varies in width from about 10 to 40 μm. The total diameter of the sensillar structures observed with the SEM is thus about 40 to 100 μm—not much less, when allowance is made for tissue shrinkage, than the sensilla diameters measured with the dissecting microscope. Thus, the central region and its surrounding ring observed with the SEM probably correspond directly to the grey discs seen with the light microscope.

Figure 6 shows at higher magnification the sensilla in Figure 5. The central region is obvious as a raised area and the white projections appear as filiform processes from the central region.

Filiform projections

Filiform projections from leech sensilla are known from previous studies with the light microscope. Illustrations from Mann (1961) indicate that these projections are found only at the central area of a sensillum, as our SEM micrographs also indicate. As Figure 7 shows, there are two distinct types of filiform processes: some are long and occur singly; others are shorter and occur as groups of two or more subunits. We have labeled the single projections "S-hairs" and the grouped structures "G-hairs." Both types appear to be evenly distributed over the central region without segregation by type. The S-hairs appear bent, suggesting flexibility, while the G-hairs have a more rigid, rod-like appearance.

The hairs' detailed structure is more evident at the high magnification of Figure 8. The S-hairs taper from a diameter of about 0.4 μm where they leave the body wall to a diameter of about 0.2 μm at their tips. The S-hairs at the ventral sensilla (at S1 and S2) are relatively short (1–3 μm long). At the more dorsal sensilla (S3–S7), these hairs are longer (4–9 μm). Because of foreshortening from the projection of a three-dimensional object onto a two dimensional surface, these measured lengths are minimum values. The G-hairs derive their appearance from the close apposition of two or more projections that individually resemble short S-hairs. The length of G-hairs at all sensilla is nearly equal (1–2 μm), considerably shorter than the dorsal S-hairs but only slightly shorter than the ventral S-hairs.

The hairs are not distributed uniformly. The ventral-most sensillum, S1, has 89 (SD = 10, N = 8) S-hairs and 18 (SD = 5, N = 8) G-hairs, S2 has 60 (SD = 9, N = 9) S-hairs and 17 (SD = 4, N = 9) G-hairs, S3 has 54 (SD = 7, N = 7) S-hairs and 20 (SD = 7, N = 7) G-hairs, and S4–S7 (dorsal sensilla) have 40 (SD = 22, N = 14) S-hairs and 16 (SD = 9, N = 14) G-hairs. Thus, while the number of G-hairs per sensillum is nearly constant, there are more S-hairs per sensillum on the ventral body wall.

Discussion

Two new aspects of the external morphology of leech sensilla are reported here. First, the SEM photomicrographs show that the sensilla have two distinct substructures: a central raised region with a diameter of about 20 μm, which bears numerous filiform projections; and a raised ring about 10–40 μm wide surrounding this central region. In contrast to the general body wall, the sensilla are devoid of pores. Second, the filiform processes observed at the sensillum fall into two distinct classes: single, relatively long hairs (S-hairs) and shorter hairs found in groups of two or more (G-hairs).

The central regions of leech sensilla resemble in size and general appearance the earthworm "sensory buds" described by Moment and Johnson (1979). In both of these annelids, these sensory structures are round, raised areas bearing numerous projections. And in both, these structures encircle the midbody regions on regularly spaced annuli. The sensilla of the medicinal leech, however, have two types of projections, while only one type of projection, single hairs, has been observed at the sensory buds of the earthworm (*Eisenia foedita*) (Moment and Johnson, 1979). The earthworm's single hairs resemble more closely the individual component projections of the short leech G-hairs than the longer, single S-hairs.

Recent physiological experiments (Young, Dedwylder, and Friesen, in press) have confirmed earlier studies by Herter (1929) that leeches are responsive to water vibrations. In particular, it was found that quiescent, submerged leeches will initiate locomotory activity in response to the stimulation provided by surface water waves. In the leech midbody, sensitivity to such wave stimulation is confined to the sensilla (Friesen, 1981). The filiform projections on the leech body wall are the best candidates for the receptors mediating vibration sensitivity, both because of their location at the sensilla and because of their similarity to other filiform processes known or suspected to be vibration receptors in vertebrates (Flock, 1965) and invertebrates (Tautz, 1979). Of the two hair types, the longer S-hairs appear better suited for transmitting the stimulus provided by the movements of water particles to sensory neurons.

Because of boundary layer effects, the amplitude of vibratory movements of water particles within 10 μm of the leech body wall is very small. For a filiform sensory structure to transmit vibrational energy effectively from the medium to the neuronal transducer, it must project beyond the stationary boundary layer (Tautz, 1979). Thus, the leech S-hairs, which appear no longer than 10 μm, must extend beyond the boundary layer to serve as sensory structures. This problem appears to have been solved in the leech by placement of the hairs at the sensilla, papillar structures that can be protruded from the body surface (Mann, 1961). In the medicinal leech, the sensilla can protrude 50 μm or more from the body wall (personal observations). Thus, the S-hairs appear to be suited for the role of water movement receptors.

ACKNOWLEDGMENTS

Supported by NIH grant NS 14965.

LITERATURE CITED

Blackshaw, S., and J. Nicholls. 1979. Distribution and morphology of touch cell endings in leech skin. *J. Physiol.* **292:** 26P–27P.

Desser, S. S., and I. Weller. 1977. Ultrastructural observations on the body wall of the leech, *Batracobedella picta. Tiss. Cell* **9:** 35–42.

Flock, A. 1965. Electron microscopic and electrophysiological studies on the lateral line canal organ. *Acta Oto-Laryn. Suppl.* **199:** 1–90.

Friesen, W. O. 1981. Physiology of water motion detection in the medicinal leech. *J. Exp. Biol.* **63:** In press.

Hayat, M. A. 1978. Pp. 134–136 In *Introduction to biological scanning electron microscopy.* University Park Press, Baltimore.

Herter, K. 1929. Reizphysiologisches Verhalten und Parasitismus des Entenegels *Protoclepsis tesselata* O. F. Mull. *Z. Vergl. Physiol.* **10:** 272–308.

Kretz, J., G. S. Stent, and W. B. Kristan. 1976. Photosensory input pathways in the medicinal leech. *J. Comp. Physiol.* **106:** 1–37.

Kristan, W. B., G. S. Stent, and C. A. Ort. 1974. Neuronal control of swimming in the medicinal leech. I. Dynamics of the swimming rhythm. *J. Comp. Physiol.* **94:** 97–119.

Mann, K. H. 1961. *Leeches.* Pergamon Press, London. 201 pp.

Moment, G. B., and E. Johnson, Jr. 1979. The structure and distribution of external sense organs in newly hatched and mature earthworms. *J. Morphol.* **159:** 1–16.

Nicholls, J. G., and D. A. Baylor. 1968. Specific modalities and receptive fields of sensory neurons in the CNS of the leech. *J. Neurophysiol.* **31:** 740–756.

Tautz, J. 1979. Reception of particle oscillation in a medium—an unorthodox sensory capacity. *Naturwissenshaften* **66:** 452–461.

Whitman, C. O. 1886. The leeches of Japan. *J. Micr. Sci. N.S.* **26:** 317–416.

r3

Reference: *Biol. Bull.* **160:** 394–418. (June, 1981)

DISTRIBUTION PATTERNS OF COMMON SEASTARS OF THE MIDDLE ATLANTIC CONTINENTAL SHELF OF THE NORTHWEST ATLANTIC (GULF OF MAINE TO CAPE HATTERAS)

D. R. FRANZ, E. K. WORLEY, AND A. S. MERRILL[1]

Biology Department, Brooklyn College, CUNY, Brooklyn, N. Y. 11210

ABSTRACT

Twenty-five asteroid species were collected during four groundfish surveys carried out by the National Marine Fisheries Service on the continental shelf from the Gulf of Maine to Cape Hatteras. Six species groups were identified, based on similarities in geographic ranges, bottom-temperature relationships, and depth distributions.

Species endemic to the boreal Northwest Atlantic include a group of middle- to outer-shelf species usually found in deeper waters north of Cape Hatteras, and a group of species with broad depth ranges whose inshore depth limits decrease from north to south (the phenomenon of submergence).

Seastars found only in the Gulf of Maine in this survey include a group of subarctic species (which do not live in arctic waters), and a group of arctic-subartic species (which may live in arctic waters). The latter are wider-ranging, occurring throughout the Arctic Ocean and into the North Pacific. Seasonally variable bottom isotherms associated with Georges Bank restrict the geographical distribution of the boreal and arctic-subarctic faunal groups of seastars.

Speculations are presented concerning when and where these groups originated. The possible effects of competition between *Asterias forbesi* and *A. vulgaris* on their distribution patterns are discussed.

INTRODUCTION

The general distribution patterns of the seastars of the Middle Atlantic continental shelf of eastern North America were established by A. E. Verrill (1878, 1894, 1895). H. L. Clark (1904) and W. R. Coe (1912) provided additional information on the echinoderms of southern New England. More recently, Gray *et al.* (1968) published a detailed account of the distributions of seastars of the North Carolina continental shelf, and Grainger (1964, 1966) contributed a definitive study of the seastars of Canadian arctic and subarctic coastal waters. The present report documents the distribution patterns of asteroids on the middle Atlantic continental shelf from the Gulf of Maine to Cape Hatteras, North Carolina, and provides further information on the general distribution patterns of common seastars.

This investigation also sought distribution patterns based on extended geographical ranges, depth distributions, and correlations with bottom temperatures. Such patterns identify groups of species with similar latitudinal and depth distributions. In this paper and elsewhere (Franz and Merrill, 1980a, b) we propose that these groups may be explained in terms of common origins in space and time. We

Received 19 January 1981; accepted 20 March 1981.

[1] National Marine Fisheries Service, Woods Hole, MA 02543

hope eventually to compare the distribution patterns of asteroids with those of other invertebrate taxa (*e.g.* Mollusca) that are represented adequately in the fossil record, to test hypotheses on the origins of faunal groups.

We suggest that distribution patterns of groups of seastars in the Northwest Atlantic are determined primarily by temperature and (less importantly) by other environmental factors, but that the ultimate causes probably are paleobiogeographical. With some exceptions, seastars that live north of Cape Hatteras appear to be most closely related to the fauna of the North Pacific. Certain aspects of present distribution patterns may be related to the periods of transarctic migration, *i.e.* the amount of time species have had to become adapted to the Northwest Atlantic environment.

Materials and Methods

Seastars were collected during four research cruises by personnel of the National Marine Fisheries Service (formerly the Bureau of Commercial Fisheries): R/V Delaware Cruise 60-7, A Sea Scallop Survey, Block Island to Cape Hatteras, 11-21 May 1960; R/V Delaware II, Cruise 75-19, A Winter Groundfish Survey from Martha's Vineyard to Cape Hatteras, 1–18 December 1975; R/V Delaware II, Cruise 78-01, A Surf Clam Survey from Long Island to Cape Hatteras, 4 January to 11 February 1978; R/V Delaware II, Cruise 79-03; and R/V Albatross IV, Cruise 79-04, A Groundfish Survey from the Gulf of Maine to Cape Hatteras, 26 March to 26 May 1979.

Bottom samples were taken with a standard (3.05 m) sea scallop dredge on the 60-7 Cruise, and a hydraulic surf clam dredge with a 1.2 m (48 in.) knife on Cruise 78-01. On Cruises 75-19, 79-03, and 79-04, seastars were collected by standard otter trawls. All seastars were bagged and frozen immediately. Asteroids from Cruise 60-7 were identified by Dr. M. J. Cerame-Vivas (University of Puerto Rico). Other collections were sorted and identified by the authors and verified by M. E. Downey (United States National Museum of Natural History, where selected samples of these animals have been deposited).

For all of the species encountered in this study, we have indicated the bottom temperatures that most closely correspond with their geographical distributions. Average minimum bottom temperatures occur in February and average maximum temperatures in September. The bottom temperature data are from Colton and Stoddard (1973) for the continental shelf from Nova Scotia to New Jersey, and from Walford and Wickland (1968) for the area from Long Island to Cape Hatteras, North Carolina, supplemented by Bowman and Wunderlich (1977) for the New York Bight. These correlations do not demonstrate thermal limits decisively, although they do indicate an approximate range of critical temperatures that may determine distribution.

Biogeographical terminology

In describing the ranges of cold-water seastars, we use the terms arctic and subarctic as defined by Dunbar (1951). The arctic zone is characterized by bottom temperatures near freezing (−1.7°C) throughout the year. The subarctic zone is a region of mixed arctic and Atlantic water entirely free of sea ice in the summer, in which bottom temperatures are usually 2°C. In the North Atlantic, the subarctic zone extends from Newfoundland to the Barents Sea, including the Labrador and West Greenland continental shelves (Dunbar, 1953). Temperature-salinity diagrams for this area (Dunbar, 1951) show that at depths greater than 50 m, temperatures range seasonally from *ca.* 1° to 7°C. Bottom temperatures remain less than 7°C throughout the year in the Gulf of Maine (Colton and Stoddard, 1973) except near shore and in the southwestern region, where seasonal contrasts are somewhat greater. Thus, we include the major basins of the Gulf of Maine as part of the subarctic zone.

The boreal zone of the European North Atlantic was defined originally as the area of the continental shelf inhabited by the cold-temperate fauna of the Northern European coast (Ekman, 1953). A significant number of these species (the amphiatlantic fauna) have ranges which extend into the Northwest Atlantic. Here, the boreal zone is defined as that portion of the continental shelf delimited by the ranges of these amphiatlantic species *i.e.* Labrador to Cape Hatteras. Some boreal species have restricted ranges within these boundaries, and some may extend somewhat farther north. The boreal species discussed in this paper have ranges which include the continental shelf from the eastern tip of Georges Bank to Cape Hatteras, excluding the Gulf of Maine. Some species which have arctic or subarctic distributions follow cold isotherms south of latitude 42°N down the continental slope, but are not found on the shelf. These species are not considered to be in "boreal waters" as defined above.

Hall (1964), Sanders (1968, 1969), and others emphasized the differences in the marine climates of the two Atlantic boreal zones. Temperature fluctuations are more rapid and seasonally more extreme on the American continental shelf than in the Northeastern Atlantic. On Georges Bank, for example, bottom temperatures may range seasonally from 4°–16°C (Colton and Stoddard, 1973) as compared to 9°–17°C near the southwestern entrance to the English Channel (Ekman, 1953).

RESULTS

The 24 species included in this report are presented in the following categories: (1) subtropical-tropical continental shelf species; (2) Atlantic coast species ranging extensively both north and south of Cape Hatteras; (3) Northwest Atlantic outer-shelf boreal species; (4) Northwest Atlantic subarctic-boreal species; (5) subarctic species; (6) arctic-subarctic species.

Group 1: Subtropical–tropical continental shelf species (Table I)

Of the five subtropical-tropical species encountered in this study, only *Luidia clathrata* and *Astropecten articulatus* were collected on the inner and middle shelf near and north of Cape Hatteras (Figs. 1, 2). Both species were found in progressively deeper sites north of Cape Hatteras (Figs. 17, 18). The northward distributions of *A. articulatus* and *L. clathrata* roughly follow the average position of the 10°C winter bottom isotherm, but do not correlate with summer bottom temperatures north of Cape Hatteras (Figs. 3, 4). The northward submergence of these species may reflect warm bottom isotherms associated with the outer continental shelf north of Cape Hatteras, as Hazel (1970) suggested to explain patterns in benthic ostracods.

Stephanasterias gracilis (Figs. 2, 18) was found at four sites along the outer continental shelf northeast of Cape Hatteras over a depth range of 106–146 m (Cruise 60-7 only). This corresponds to the northward range limits reported by Gray *et al.* (1968). Single specimens of both *Narcissia trigonaria* (not mapped) and *Luidia alternata* (not mapped) were collected at stations south of Cape Hatteras (Table I).

Group 2: Atlantic Coast species that range extensively both north and south of Cape Hatteras

Only one species in this survey, *Asterias forbesi*, ranges extensively both north and south of Cape Hatteras. It is found from Casco Bay, Maine (based on records in the United States National Museum) south to the Florida Keys, although it is evidently rare south of latitude 28°N (Downey, 1973).

In the zone from Cape Hatteras to Cape Cod (Fig. 5), *A. forbesi* was distributed over the entire continental shelf, except north of 40°N, where collections were largely confined to the inner shelf, on Nantucket Shoals, and in Cape Cod Bay (in agreement with Clark, 1904). Despite its distribution over much of the continental shelf, *A. forbesi* was collected most frequently at stations less than 30 m in depth (Fig. 21). The relationship between bottom temperatures and distribution is discussed below, in conjunction with *A. vulgaris*.

Group 3: Northwest Atlantic outer shelf boreal species (Table II)

The four species composing the Northwest Atlantic outer shelf boreal species, *Astropecten americanus, Sclerasterias tanneri, Porania insignis*, and *Odontaster hispidus*, are restricted to the middle and outer continental shelf (Georges Bank to Cape Hatteras) (Table II), at depths greater than 50 m (with the exception of *A. americanus*). Both *A. americanus* and *S. tanneri* exhibit northward submergence.

A. americanus is found only in areas of the continental shelf where winter bottom temperatures remain above 5°–6°C (Fig. 6). These isotherms cross the 100 m contour on the outer edge of Georges Bank, south of Nantucket. Winter temperatures on the inner shelf from Georges Bank to northern North Carolina generally remain below 7.5°C, probably accounting for the absence of *A. americanus*. Summer bottom temperatures coinciding with the geographical distribution of *A. americanus* range from about 10°C (off Georges Bank) to about 20°C on the outer shelf near Cape Hatteras.

The distribution of *A. americanus* may also be limited by warm summer temperatures above 20°C, possibly accounting for its absence from the inner continental shelf off northern North Carolina and from almost the entire shelf south of Cape Hatteras. Near to and south of Cape Hatteras, *A. americanus* is replaced by *A. articulatus*. Interactions between these species in relation to environmental factors, and especially to seasonal marine climate, should be investigated.

A. americanus (Fig. 19) is found in progressively deeper waters northward. Its upper depth limit declines gradually from about 25 m near Cape Hatteras to about 50 m at latitude 40°30′N, followed by a sharper decline northward to 149 m near its northern range limit on Georges Bank. As with *A. articulatus* and *Luidia clathrata*, this northward submergence implies the tracking of warmer winter isotherms into deeper waters.

The distribution of *Sclerasterias tanneri* (Figs. 7, 20) is qualitatively similar to *A. americanus* except that *S. tanneri* is more strictly confined to the outer continental shelf and to the upper slope. This species occurs where winter bottom

TABLE I

Subtropical/tropical species—present survey.

Species	Geographical range	Reference	Northern limit	Depth range (m)	Previously known depth range (m)
Astropecten articulatus (Say)	Off Chesapeake Bay to Columbia	Downey (1973)	36°23′ 74°54′	18–44	18–165
Luidia clathrata (Say)	New Jersey* to Brazil	Downey (1973)	37°06′ 74°48′	18–74	0–175
Stephanasterias gracilis Verrill	North Carolina to Lesser Antilles	Gray *et al.* (1968)	36°52′ 74°36′	106–146	145–370
Narcissia trigonaria Sladen	North Carolina to Brazil	Downey (1973)	34°08′ 76°12′	65	37–91
Luidia alternata (Say)	North Carolina to Brazil	Downey (1973)	34°02′ 76°25′	40	10–200

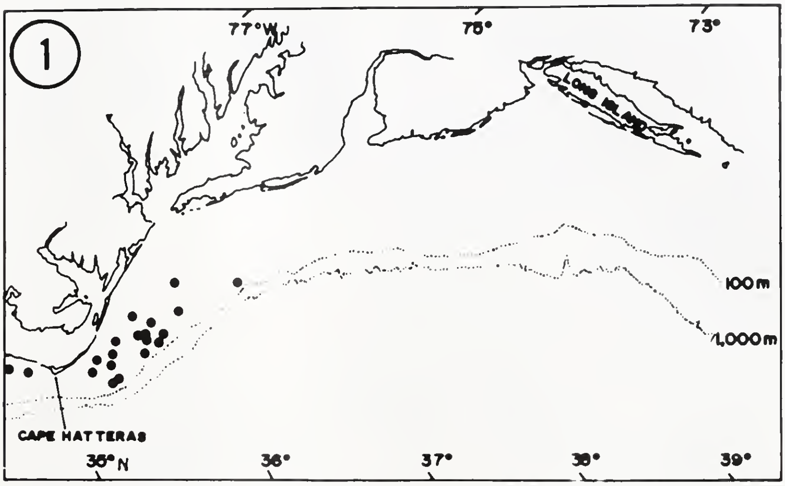
* The New Jersey record may be in error

TABLE II

Outer shelf boreal species—present survey.

Species	Geographical range	Reference	Northern limit	Southern limit	Depth range (m)	Previously known depth range (m)
Astropecten americanus Verrill	40°23′N to 35°39′N	Verrill (1895)	42°53′N, 69°18′W	35°25′N, 75°13′W	26–168	55–542
Sclerasterias tanneri Verrill	40°81′N to 35°10′N	Verrill (1895)	42°09′N, 66°26′W	36°36′N, 74°52′W	62–186	88–355
Odontaster hispidus Verrill	Cape Cod to Florida	Gray *et al.* (1968)	39°44′N, 72°04′W	36°52′N, 74°36′W	146	30–875
Porania insignis Verrill	Georges Bank to North Carolina	Verrill (1895)	43°19′N, 67°18′W	40°17′N, 68°16′W	83–256	(183–457)[1] (35–680)[2]

[1] Verrill, 1895
[2] Gray *et al.*, 1968



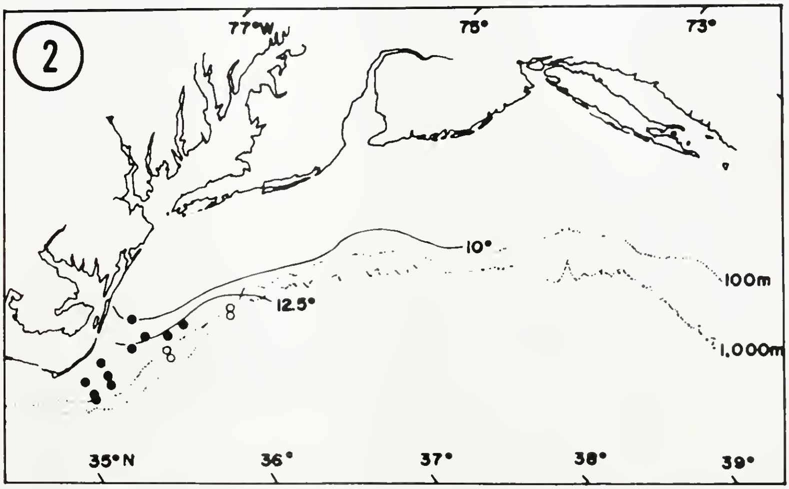


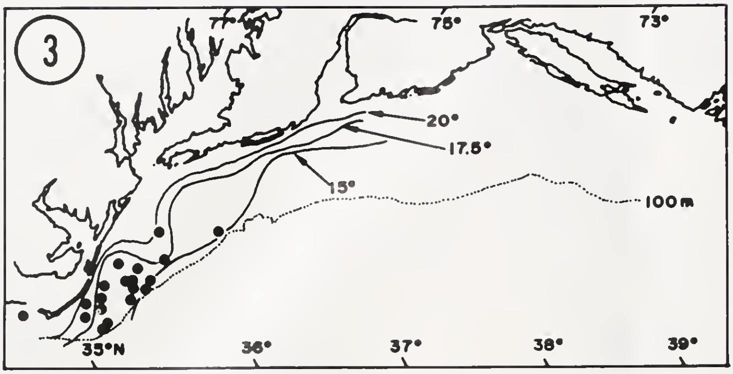
FIGURE 1. Distribution of *Luidia clathrata* on the continental shelf off Cape Hatteras, North Carolina.

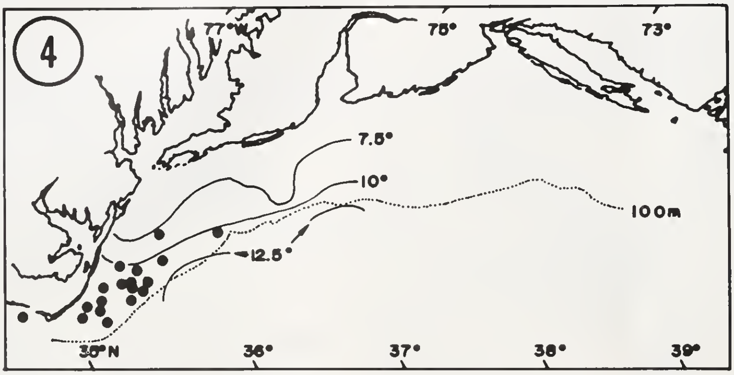
FIGURE 2. Distribution of *Astropecten articulatus* (solid circles) and *Stephanasterias gracilis* (open circles) on the continental shelf north of Cape Hatteras in relation to 10°C and 12.5°C February bottom isotherms.

temperatures remain warmer than 7°–8°C (Fig. 7), and where summer bottom isotherms probably do not exceed about 15°C (and are probably less than 10°C over much of the range). The deep-sea red crab (*Geryon quinquidens*) has a similar distribution north of Cape Hatteras, but is known to tolerate water temperatures considerably higher than those encountered in its natural environment (Wigley *et al.*, 1975). This may also be true of *S. tanneri.* It seems likely that winter bottom temperatures are more important than summer temperatures in determining the range of *S. tanneri.*

Odontaster hispidus (Fig. 7) was collected at only two stations off New Jersey and Virginia (Table II).

Porania insignis was taken at a scattering of stations in the central and western


3
77°
75°
73°
20°
17.5°
15°
100m
35°N
36°
37°
38°
39°


4
77°W
75°
73°
7.5°
10°
12.5°
100m
35°N
36°
37°
38°
39°

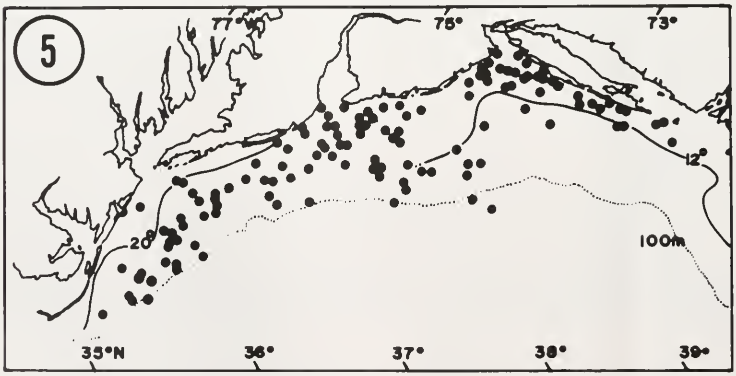
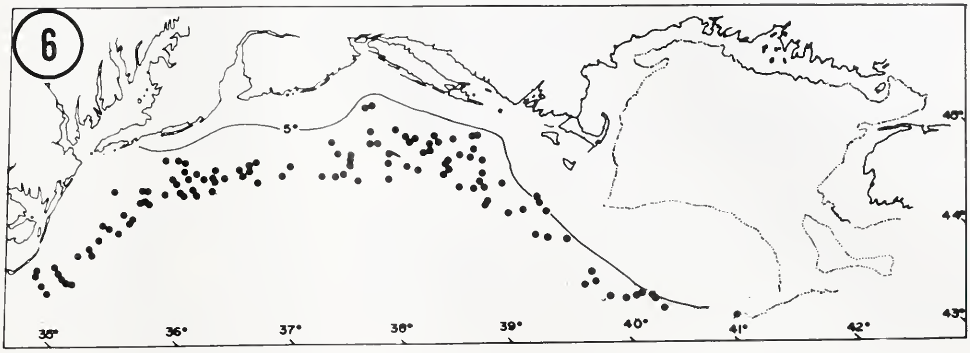

5
77°W
75°
73°
12°
20°
100m
35°N
36°
37°
38°
39°

FIGURE 3. Distribution of *Luidia clathrata* in relation to September bottom isotherms, showing absence of correlation between summer temperatures and distribution. This pattern is very similar to *Astropecten articulatus*.

FIGURE 4. Distribution of *Luidia clathrata* in relation to February bottom isotherms. *L. clathrata* collections come from areas where bottom temperatures in winter remain above 7–10°C, a pattern very similar to *Astropecten articulatus*.

FIGURE 5. Distribution of *Asterias forbesi* on the Middle Atlantic continental shelf in relation to the 12° and 20°C September bottom isotherms. *A. forbesi* was sparse inshore of the 20° isotherm south of Virginia (where temperatures may reach or exceed 25°C). Bottom temperatures above 12°C extend northward to Nantucket Shoals and Georges Bank (Fig. 25) although *A. forbesi* is limited to the shelf south of Cape Cod.



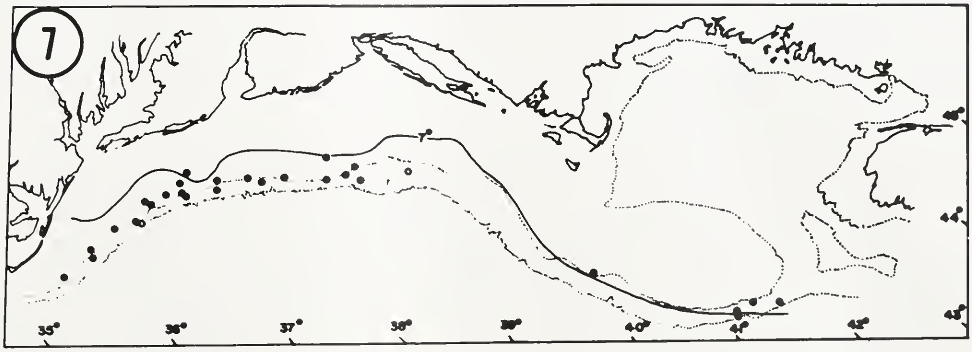


FIGURE 6. Distribution of *Astropecten americanus* in relation to the 5°C February bottom isotherm. Inshore of this isotherm, winter temperatures are less than 5°C.

FIGURE 7. Distribution of *Sclerasterias tanneri* (solid circles) and *Odontaster hispidus* (open circles) in relation to the 7°C February bottom isotherm. Seaward of this isotherm, winter bottom temperatures exceed 7°C.

Gulf of Maine and at the eastern tip of Georges Bank (Fig. 15). Its depth and geographical distribution patterns (Table II) indicate that *P. insignis* is an outer shelf/upper slope species that may track continental slope water into the Gulf of Maine via the Northeast Channel. Fluctuation in bottom temperatures in the observed range of this species is from about 5°C (winter) to around 10°C (summer). Since winter bottom temperatures on the lower shelf and upper slope over the geographical range of *P. insignis* (Georges Bank to North Carolina) range from roughly 8°–12°C, the upper depth limit and northern range limit of this species in the Gulf of Maine probably are determined by winter temperatures in the range of 5°–6°C. Whether or not *P. insignis* is able to reproduce at these temperatures is unknown.

Group 4: Northwest Atlantic subarctic-boreal species (Table III)

The species in the northwest Atlantic subarctic-boreal group, *Asterias vulgaris*, *Leptasterias tenera*, and *Henricia* spp., are confined to the subarctic-boreal zone on the eastern American coast. Their upper (inshore) depth limits become deeper with decreasing latitude (boreal submergence). These species are either very closely

TABLE III

Subarctic-boreal species—present survey.

Species	Geographical range	Reference	Northern limit	Southern limit	Depth range (m)	Previously known depth range (m)
Asterias vulgaris Verrill	Gulf of St. Lawrence (excluding the Strait of Belle Isle) to off Cape Hatteras, North Carolina	Grainger, 1964 Verrill, 1895	44°37′N, 66°10′W	35°22′N, 74°55′W	15–348	0–358
Leptasterias tenera (Stimpson)	45°29′ to 37°19′N[1,2]	Verrill, 1895	42°57′N, 65°23′W	35°22′N, 74°55′W	31–182	18–236
Henricia spp.	47°29′N to 35°38′N on the coast of the U. S. but extending north into arctic waters[3]	Verrill, 1895	44°37′N, 66°10′W	36°52′N, 74°48′W	33–196	0–662[3]

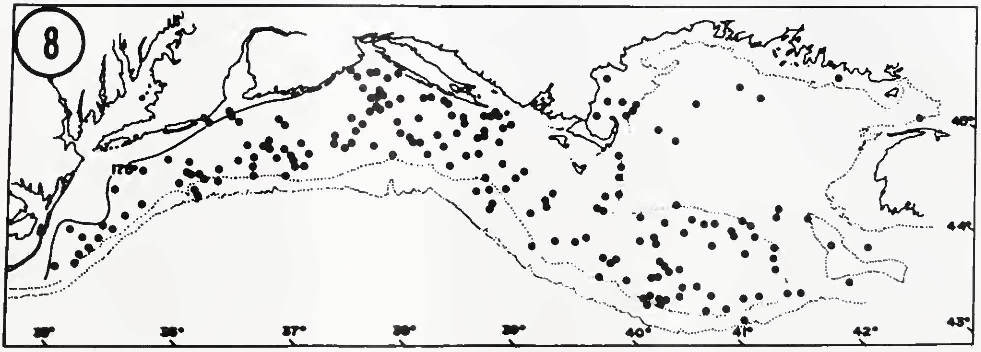
[1] Including *L. compta*, which is generally considered synonymous with *L. tenera*

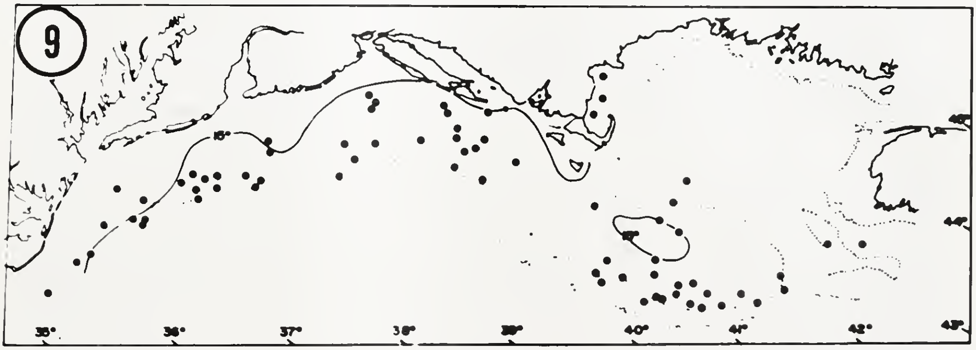
[2] To southern tip of Greenland according to Mortensen (1932), who suggested that this may represent a recent northern range extension

[3] For *H. sanguinolenta*

related to Northeastern Atlantic congeners (*e.g. Leptasterias tenera* and *L. mulleri*), or are synonymous with European species, *e.g. A. vulgaris* with *A. rubens; Henricia* spp. (in part) with *H. sanguinolenta.*

A. vulgaris is evidently morphologically indistinguishable from the European *A. rubens*, justifying the synonymizing of these species (M. E. Downey, personal





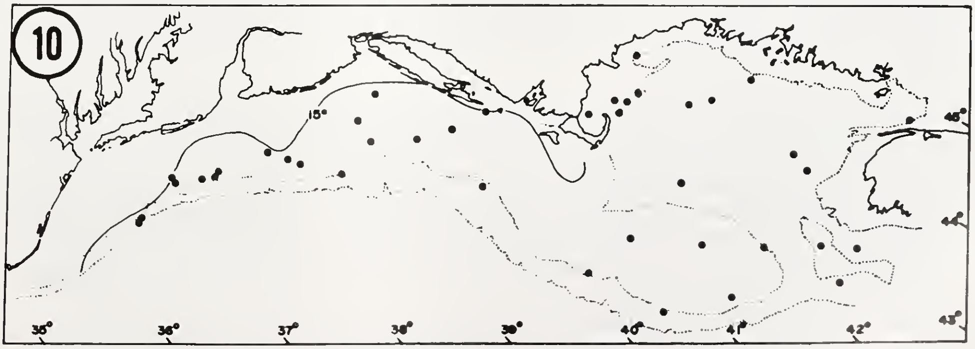


FIGURE 8. Distribution of *Asterias vulgaris*, Gulf of Maine to Cape Hatteras. South of latitude 38°N, distribution is correlated with the 17.5°C bottom isotherm. Inshore of this isotherm, summer bottom temperatures may exceed 17°C.

FIGURE 9. Distribution of *Leptasterias tenera*, Gulf of Maine to Cape Hatteras, in relation to the 15°C September bottom isotherm. Inshore of this isotherm summer bottom temperatures exceed 15°C.

FIGURE 10. Distribution of *Henricia* spp., Gulf of Maine to Cape Hatteras, in relation to the 15°C September bottom isotherm. Inshore of this isotherm summer bottom temperatures exceed 15°C.

communication). However, populations from the Northwest Atlantic may be ecologically and genetically isolated from populations in Iceland and the Northeast Atlantic. Therefore, we have continued to use the name *vulgaris* for American Atlantic populations of *A. rubens.*

A. vulgaris (Figs. 8, 22) occurs over entire breadth of the continental shelf in the zone covered in this survey, from the entrance to the Bay of Fundy south to Maryland. Further south, it is confined to the middle and outer shelf, reaching its southern limit off Cape Hatteras (Table III). The upper (inshore) vertical limit is correlated with summer bottom isotherms of about 17°C. Thus, it would appear that permanent populations of *A. vulgaris* are not found where average summer bottom temperatures exceed this temperature. Winter bottom temperatures corresponding to the observed geographical range vary from near 15°C (off Cape Hatteras) to less than 3°C (Cape Cod Bay, portions of the western Gulf of Maine).

The mechanisms by which temperature controls the southern limits of *A. vulgaris* have not been adequately investigated. In eastern Canada, *A. vulgaris* stops feeding in midsummer (Needler, 1941), and dies when temperatures reach 25°C (Smith, 1940). Clearly, *A. vulgaris* can tolerate temperatures between 17°C and 25°C at least for short periods of time.

The thermal requirements for reproduction of *A. vulgaris* are imperfectly understood, although it is generally recognized as a late spring and summer spawner, with larvae settling from midsummer to autumn (Boolootian, 1966). If all of the points in Figure 22 represent reproducing populations, *A. vulgaris* can occur where summer bottom temperatures reach as low as 6°C. If the records of *A. vulgaris* from the deeper basins of the Gulf of Maine, where bottom temperatures remain at or below 6°C in summer, represent non-breeding animals, the northern limit of *A. vulgaris* would be more closely correlated with summer bottom temperatures of about 8°C. This corresponds to the temperature at which *A. rubens* begins spawning in the White Sea, near the northern range limit of this species (Mileikovsky, 1970). Thus, while the northern limit of *A. vulgaris* may be determined by minimum summer bottom temperatures in the range of 6°–8°C, it is not possible to be more precise, and the effects of temperature on growth, spawning, post-larval development, etc. are unknown. According to Grainger (1964), *A. vulgaris* reaches its northern geographical limits in the Gulf of St. Lawrence, south of the Strait of Belle Isle. Analysis of distribution in relation to seasonal bottom temperatures of this northern population would provide more definitive information on the thermal control of distribution.

Leptasterias tenera is widely distributed on the middle and outer continental shelf from near Cape Hatteras to Brown Bank (off Nova Scotia), and the inner shelf off eastern Long Island, and in Cape Cod Bay (Fig. 9, 23). Hendler and Franz (in preparation) found that in Cape Cod Bay and Block Island Sound, *L. tenera* broods its young from December through March, at bottom temperatures which range from about 3°–7°C. In Block Island Sound, the winter brooding period is followed by a very active feeding period which, however, declines as bottom temperatures rise above 10°–12°C.

The distribution of *L. tenera* is fairly well correlated with the 15°C mean September bottom isotherm (Fig. 9). Average winter bottom isotherms over this area range from 3°–12°C. Thus, warm summer temperatures, probably affecting feeding rate, appear to limit the distribution of *L. tenera.* Since *L. tenera* occurs on the outer shelf and upper slope northeast of Cape Hatteras, it must brood over a large winter temperature range—roughly from 3°–12°C. However, the role of cold winter temperatures in controlling its northward distribution is unclear. Hendler and Franz (in preparation) noted that the feeding rates of both sexes were lower during January and February. In the Gulf of Maine, *L. tenera* is largely confined to areas in which summer bottom temperatures reach 7°–8°C.

L. tenera is closely related to the European *L. mulleri*, and according to Fisher (1930), at least some of the northwestern Atlantic records of *L. mulleri* may be this species. Grainger (1966) suggested that some specimens identified as *L. mulleri* from the Canadian Atlantic coast may in fact be *L. floccosa*. Clearly, the distribution of *L. tenera* in Canadian Atlantic waters needs to be re-evaluated.

The genus *Henricia* presents confusing nomenclatural and taxonomic problems. *H. sanguinolenta*, a taxon which has been used as a catchall for what may be several Northwest Atlantic species (Grainger, 1966), is a common intertidal species in New England, but undergoes a marked submergence southward (Coe, 1912).

The distribution of *Henricia* spp. (Figs. 10, 24) includes the middle and outer continental shelf from the Gulf of Maine and Georges Bank to off the Virginia coast. The upper (inshore) depth limit drops gradually from north to south, reaching about 50 m at latitude 38°N, and then dropping precipitously to about 150 m at its southern limit. This suggests that the distribution of *Henricia* is limited to areas where summer bottom temperatures do not exceed about 15°C (Fig. 10).

Group 5: Subarctic species (Table IV)

The predominantly subarctic seastars encountered in this survey (Figs. 11–13) were *Hippasteria phygiana, Ceramaster granularis, Poraniomorpha hispida, Psilaster andromeda, Leptychaster arcticus,* and *Pseudarchaster parelii.* None of these occurs in arctic waters; in the present study, all were confined to the Gulf of Maine.

These subarctic species extend into very deep waters, although three species were collected in the Gulf of Maine at depths as shallow as 35 m (Table IV). Since the southern geographical limits of all six species are in the Gulf of Maine (excluding continental slope records), thermal limits probably are reached in this area. Summer bottom temperatures corresponding to the distribution patterns of all of the above species do not exceed 7°–8°C, with winter minimum temperatures in the range of 4°C–6°C. Since winter bottom temperatures on nearby Georges Bank are the same as those in the Gulf of Maine, the presence of these species in the Gulf, and their absence from Georges Bank, is probably correlated with summer bottom temperatures.

Group 6: Arctic-subarctic species (Table V)

Six species of seastars, all collected from the Gulf of Maine in this survey, are widely distributed in both arctic and subarctic waters: *Crossaster papposus* (Fig. 14), *Solaster endeca* (Fig. 14), *Ctenodiscus crispatus* (Fig. 15), *Pontaster tenuispinus* (Fig. 15), *Pteraster militaris* (Fig. 16), and *Lophaster furcifer* (Fig. 16).

Both *Crossaster papposus* and *Solaster endeca* occurred in relatively shallow waters compared to other arctic-subarctic species (Table V). *C. papposus* is a predator mostly on asteroids (Feder and Christensen, 1966) and especially on *Asterias* (Mortensen, 1927), and inhabits a wide range of sediments (Grainger, 1966). The concentration of this species on the edge of the continental shelf off Massachusetts Bay, and the eastern tip of Georges Bank (Fig. 14) may reflect a higher biomass of its favored prey.

Summer maximum bottom temperatures corresponding to the observed distri-

TABLE IV

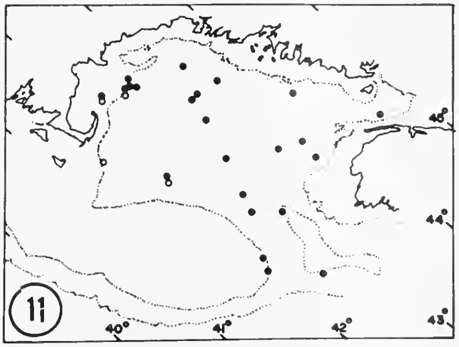
Subarctic species—present survey.

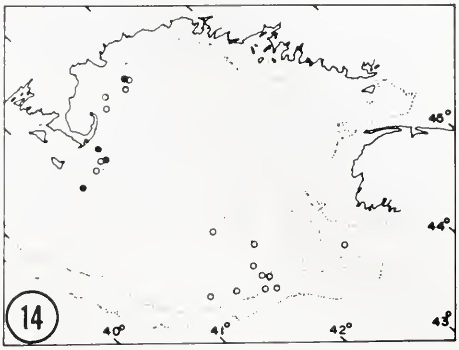
Species	Geographical range	Reference	Southern limit	Depth range (m)	Overall depth range (m)
Hippasteria phrygiana (Parelius)	Cape Cod to Nova Scotia; East and West Greenland to Scotland and England (Channel), Scandinavia from the Kattegat to Finmark	Mortensen, 1927	41°29′N, 66°26′W	35–348	20–800
Ceramaster granularis (O. F. Muller)	Gulf of Maine to Newfoundland; Southwest Greenland to the Faroes; Scandinavia from Skagerrak to Finmark; Morocco and Azores; Bering Sea	Grainger, 1966 Mortensen, 1927	42°03′N, 68°27′W	97–197	20–1400
Leptychaster arcticus (M. Sars)	Gulf of Maine to Labrador; West Greenland to Faroes; Scandinavia From Trondheim to Finmark; Bering Sea to Japan	Verrill, 1895 Mortensen, 1927	41°40′N, 69°33′W	35–196	20–1766
Poraniomorpha hispida (M. Sars)	Off Cape Cod south to 35°12′N (deep water)[1]; Southwest Greenland to the Faroes; Skagerrak to northern Norway	Verrill, 1895 Mortensen, 1927	43°17′N, 69°34′W	135–348	90–1171
Psilaster andromeda (Muller & Troschel)	38°27′N to 44°47′N[2]; Davis Strait to Ireland; Murman coast to Cape Verdi Islands and Azores	Verrill, 1895 Mortensen, 1932	41°45′N, 69°41′W	35–348	19–1850
Pseudarchaster parelii (Duben & Koren)	37°59′N to 44°26′N[3]; Southwest Greenland to Ireland, and Barents Sea; Bering Sea to N. Pacific	Verrill, 1895 Grainger, 1966	42°28′N, 66°57′W	318–348	156–2947

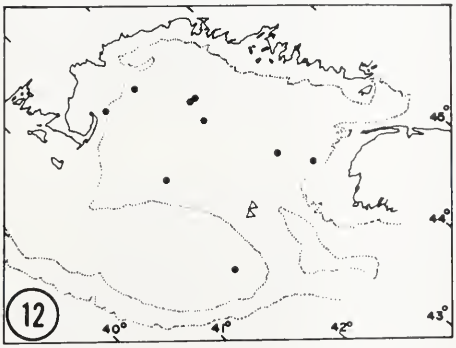
[1] As *P. spinulosa* in Verrill (1895). Verrill suggested that *P. spinulosa* was closely related to the European *P. rosea* (*P. hispida*). Mortensen (1932) agreed, as indicated by his citation of Verrill's southern limit of *P. spinulosa* in the general description of the range of *P. hispida*, but Mortensen did not actually cite *P. spinulosa* as a synonym of *P. hispida*.

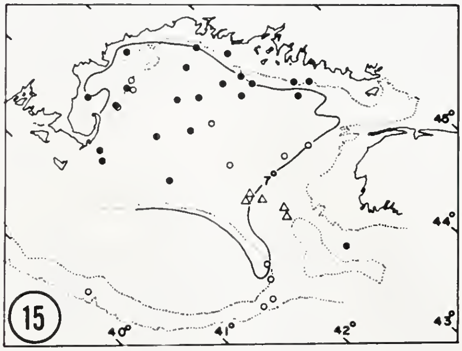
[2] In Verrill (1895) as *P. florae*

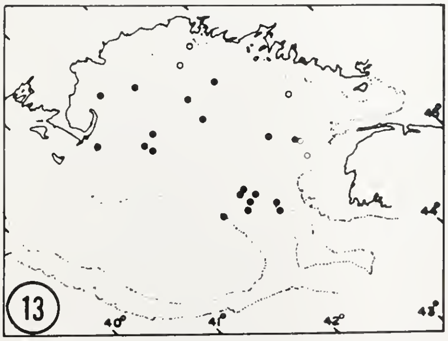
[3] In Verrill (1895) as *P. intermedius* Sladen


11
40°
41°
42°
43°
44°
45°


14
40°
41°
42°
43°
44°
45°


12
40°
41°
42°
43°
44°
45°


15
7°
40°
41°
42°
43°
44°
45°


13
40°
41°
42°
43°
44°
45°

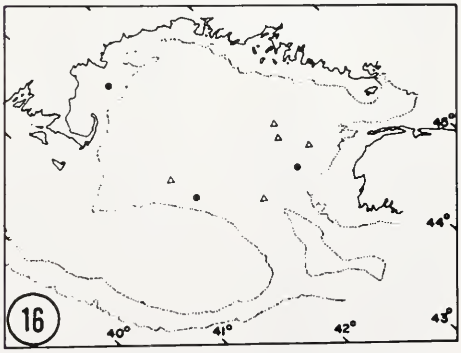

16
40°
41°
42°
43°
44°
45°

FIGURE 11. Distribution of *Hippasteria phrygiana* (solid circles) and *Leptychaster arcticus* (open circles) in the Gulf of Maine.

FIGURE 12. Distribution of *Pseudarchaster parelii* (open triangles) and *Ceramaster granularis* (solid circles) in the Gulf of Maine.

FIGURE 13. Distribution of *Poraniomorpha hispida* (open circles) and *Psilaster andromeda* (solid circles) in the Gulf of Maine.

FIGURE 14. Distribution of *Crossaster papposus* (open circles) and *Solaster endeca* (solid circles) in the Gulf of Maine.

FIGURE 15. Distribution of *Ctenodiscus crispatus* (solid circles), *Pontaster tenuspinus* (open triangles), and *Porania insignis* (open circles) in the Gulf of Maine. The 7°C September bottom isotherm is superimposed.

FIGURE 16. Distribution of *Pteraster militaris* (open triangles) and *Lophaster furcifer* (solid circles) in the Gulf of Maine.

TABLE V

Arctic–subarctic species—present survey.

Species	Range	References	Southern limit	Depth range (m)	Overall depth range (m)
Crossaster papposus (L)	North Pacific; Bering Sea, Arctic Ocean, NW Atlantic south to the Gulf of Maine; NE Atlantic south to England	Grainger, 1966	41°16′N, 66°34′W	35–186	0–1200
Solaster endeca (L)	North Pacific to Vancouver and the Okhotsk Sea; Bering Sea; Arctic Ocean; E. Canada south to Cape Cod; NE Atlantic south to England	Mortensen, 1927	41°15′N, 69°25′W	48–129	48–450
Ctenodiscus crispatus (Retzius)	North Pacific; Bering Sea; Arctic Ocean; Canadian Arctic south to Gulf of Maine; NE Atlantic south to Faroes and Trondheim	Mortensen, 1927 Grainger, 1966	41°40′N, 69°33′W	49–227	2–1900
Pontaster tenuispinus (Duben & Koren)	Arctic Ocean from NE Canada to East Siberian Sea; NW Atlantic south to Nova Scotia; NE Atlantic south to Faroes, but not North Sea	Verrill, 1895 Grainger, 1966	42°33′N, 67°17′W	154–293	20–1960
Pteraster militaris (O. F. Muller)	North Pacific south to Vancouver and Japan; Bering Sea; Arctic Canada to Chukchi Sea; NW Atlantic south to Massachusetts Bay; NE Atlantic south to Faroes and Skagerrak	Verrill, 1895 Grainger, 1966	42°03′N, 68°27′W	91–267	10–1100
Lophaster furcifer (Duben & Koren)	North Pacific (as *L.f. vexator*) to California and Japan; Arctic Ocean from NW Canada to Laptev Sea; NW Atlantic to Gulf of Maine; NE Atlantic to Faroe Channel	Verrill, 1895 Mortensen, 1927 Grainger, 1966	42°07′N, 67°55′W	49–200	30–1350

bution of *C. papposus* and *S. endeca* in the Gulf of Maine and eastern Georges Bank range from 8°–10°C. In Europe, *S. endeca* is said to tolerate summer temperatures up to 14°C (Ursin, 1960).

Ctenodiscus crispatus is a mud dweller that feeds on organic detritus (Mortensen, 1927). In the Gulf of Maine, it occurs on clay to silty sand bottoms where organic contents are probably greater than 0.5% (based on sediment data from Hayes and Wigley, 1969). Mortensen (1927) reported that *C. crispatus* could live at temperatures up to 10°C in Scandanavia. In the present study, its distribution corresponded to maximum summer temperatures of roughly 7°C (Fig. 15).

Pontaster tenuispinus and *Pteraster militaris* were restricted to relatively few, generally deep, stations in the eastern and southeastern basins of the Gulf of Maine where summer bottom temperatures remain at or below 7°–8°C. Similarly, the distribution pattern of *Lophaster furcifer* was associated with bottom temperatures not exceeding 7°–8°C. The relationship between the combined distribution patterns of all of the arctic-subarctic species recorded in this survey, and summer bottom temperatures (Fig. 25) indicates that arctic-subarctic species are not found where bottom temperatures rise much above 8°–9°C.

Discussion

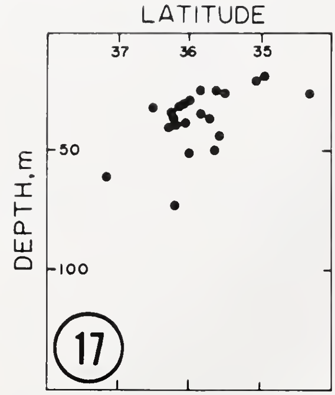
Temperature control of distribution

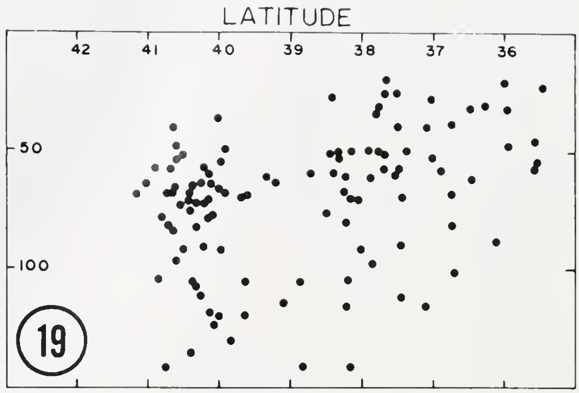
Temperature may limit the distribution of species by affecting survival, reproduction, development of juveniles, and biotic interactions (competition, predation, and disease). It may influence any stage of the life cycle (Krebs, 1978). Few of the experimental studies necessary to prove decisively that temperature limits distribution have been carried out on seastars. Independent information on the influence of temperature on physiological rates (feeding, reproduction), and survival is available for only a few of the species in the continental shelf zone of the Middle Atlantic.

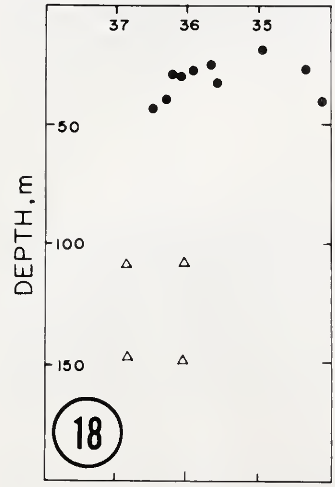
Investigations demonstrating a correlation between environmental temperature and geographical distribution are frequently used to indicate thermal control, and to suggest the ways in which temperature may act in controlling distribution. A well known model that attempts to account for temperature regulation of the latitudinal distribution of benthic marine invertebrates is that of Hutchins (1947), who proposed that temperature controls species through its effects on survival and/or reproduction (repopulation). His analysis requires the correlation of latitudinal distribution of species with seasonal minimum and maximum seawater temperatures. We have not applied Hutchins' procedures to the species distributions of seastars, primarily because the survival/reproduction dichotomy provides little information on the actual mechanisms of thermal control.

Thermal control of distribution of Asterias forbesi *and* Asterias vulgaris

Several aspects of the depth and latitudinal ranges of the common seastars *Asterias forbesi* and *Asterias vulgaris* merit further discussion, and provide the basis for speculations concerning the thermal control of distribution in these species. The feeding rate of *A. forbesi* in Long Island Sound declines above 22°C, and at 25°C animals rapidly lose weight, although feeding continues (MacKenzie, 1969). Thus, the scarcity of *A. forbesi* on the inner continental shelf off the Virginia and North Carolina coasts may be correlated with summer bottom temperatures higher than 20°C (Fig. 5).







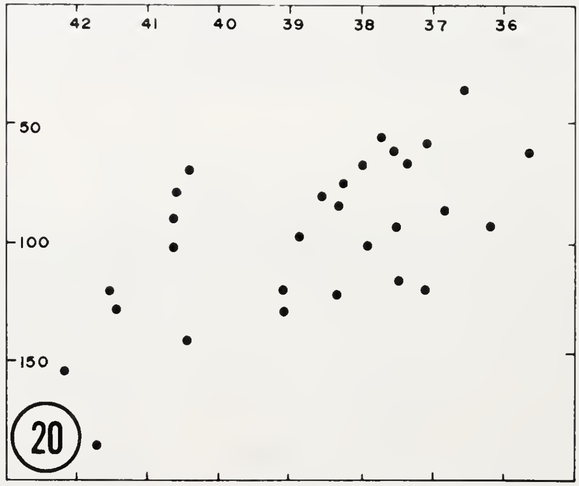


FIGURE 17. Depth distribution of *Luidia clathrata* on the continental shelf, showing northward submergence.

FIGURE 18. Depth distribution of *Stephanasterias gracilis* (open triangles) and *Astropecten articulatus* (closed circles). The latter, like *Luidia clathrata*, exhibits northward submergence.

FIGURE 19. Depth distribution of *Astropecten americanus*. The upper (inshore) depth limits undergo northward submergence.

FIGURE 20. Depth distribution of *Sclerasterias tanneri*. Like *Astropecten americanus, S. tanneri* exhibits northward submergence.

In Long Island Sound, *A. forbesi* reproduces at 15°–16°C (Galtsoff and Loosanoff, 1939; Loosanoff, 1961). If this applies to offshore populations as well, then collections in Figure 21 from the outer continental shelf, south of 40°N, may represent "pseudopopulations" in the sense of Mileikovsky (1971), *i.e.* non-breeding

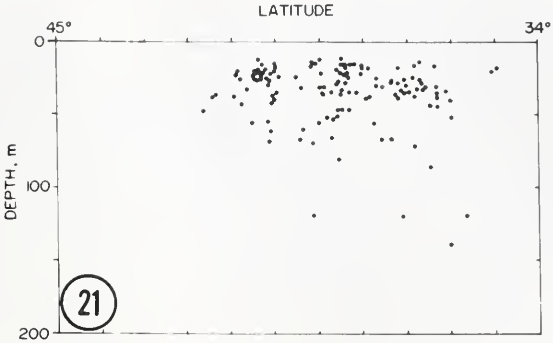
---

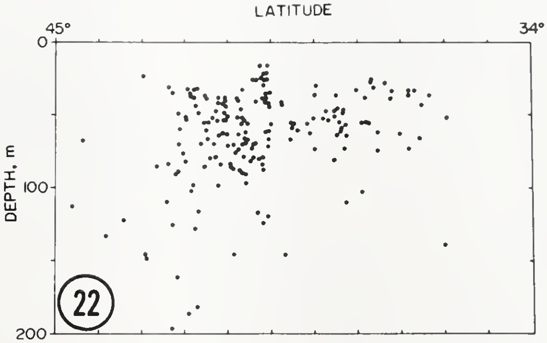
FIGURE 21. Depth distribution of *Asterias forbesi*. The upper (inshore) depth limits do not submerge to the south although the lower (offshore) depth limits increase southward.

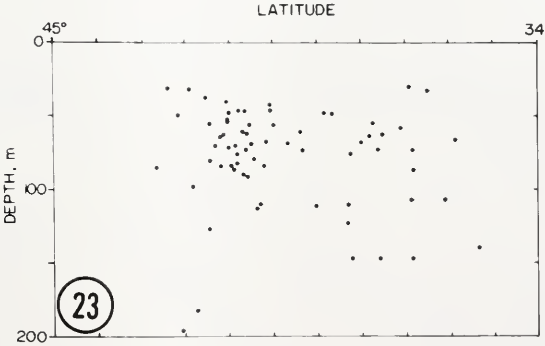
FIGURE 22. Depth distribution of *Asterias vulgaris*. The upper (inshore) depth limits undergo submergence with decreasing latitude, the phenomenon of "boreal submergence." The lower (offshore) depth limits appear to rise into shallower waters with decreasing latitude.

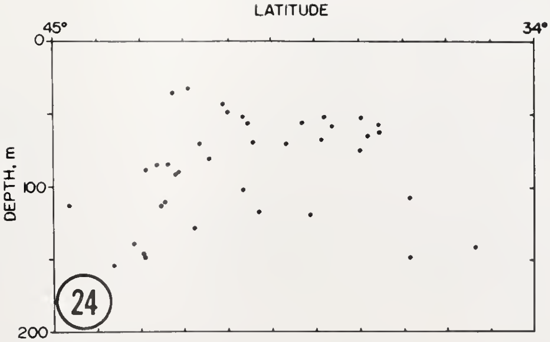
FIGURE 23. Depth distribution of *Leptasterias tenera* on the Middle Atlantic continental shelf.

FIGURE 24. Depth distribution of *Henricia spp.*, Gulf of Maine to Cape Hatteras. The upper (inshore) depth limits undergo submergence with decreasing latitude ("boreal submergence").


LATITUDE
45°
34°
0
100
200
DEPTH, m
21


LATITUDE
45°
34°
0
100
200
DEPTH, m
22


LATITUDE
45°
34°
0
100
200
DEPTH, m
23

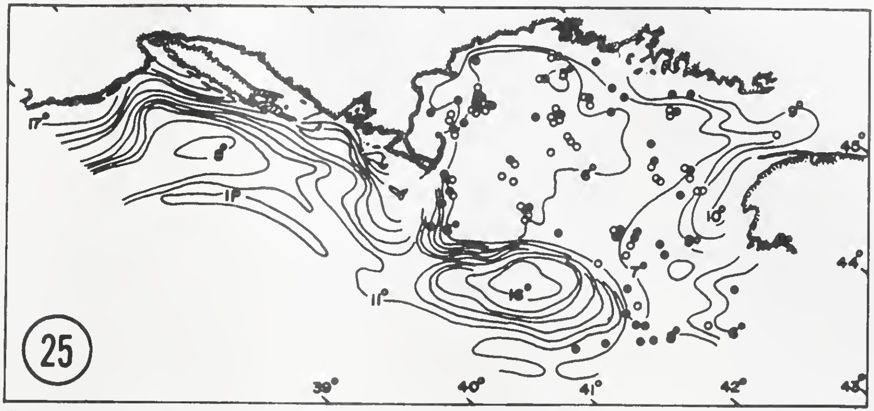

LATITUDE
45°
34°
0
100
200
DEPTH, m
24

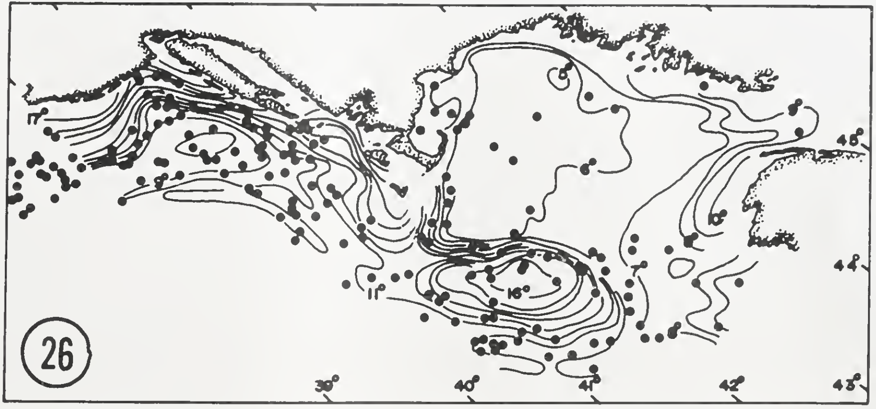
aggregations which result from larvae settling at depths where bottom temperatures remain too low for gametogenesis and/or spawning.

Bottom temperatures on portions of the inner continental shelf off eastern Long Island, Nantucket Shoals, and portions of Georges Bank approach 15°C during August and September (Colton and Stoddard, 1973). Thus, it seems likely that *A. forbesi* larvae settle in these areas. However, *A. forbesi* did not occur in our collections, although *A. vulgaris* was abundant (*cf.* Figs. 5, 8). This absence may be caused by suboptimal feeding temperatures on Nantucket Shoals and portions of Georges Bank, perhaps coupled with competition between *A. forbesi* and *A. vulgaris.* The optimum temperature for feeding in *A. forbesi* is 20°C, and feeding ceases altogether at about 5°C (Zinn, 1937; MacKenzie, 1969), whereas *A. vulgaris* probably continues to feed even at temperatures as low as 3°C (Hancock, 1955). Since winter bottom temperatures remain above 3°C but below 5°C over much of Georges Bank and Nantucket Shoals (Colton and Stoddard, 1973), *A. vulgaris* may continue feeding, a competitive advantage over dormant *A. forbesi.* Furthermore, since optimum feeding temperatures of *A. vulgaris* are probably lower than those for *A. forbesi*, bottom temperatures during the summer months would probably be more favorable for *A. vulgaris* than for *A. forbesi.* Thus, *A. vulgaris* may enjoy a competitive advantage during all seasons. If these speculations are valid, *A. forbesi* is excluded from these large and highly productive areas where, in the absence of competition, it could survive and possibly reproduce.

Depth distribution patterns support the hypothesis that interactions other than direct thermal control determine the distribution of *A. forbesi.* While *A. forbesi* is most common in shallow water (<30m) throughout its range, its lower vertical range increases southward (Fig. 21). Since bottom temperatures at intermediate depths (50–100 m) become progressively warmer with decreasing latitude, the increasing depth range may merely indicate a seaward shift in the bottom temperatures suitable to the survival of *A. forbesi.* However, as summer bottom temperatures increase, *A. forbesi* juveniles are at a lesser competitive disadvantage relative to *A. vulgaris.* Thus, nonbreeding populations of *A. forbesi* may co-exist with adult *A. vulgaris* at depths in which seasonal changes in bottom temperatures do not clearly favor either species.

The upper (vertical) depth limit of *A. vulgaris* decreases and its lower depth limit rises in a gradient from north to south (Fig. 22). A declining upper vertical limit (submergence) reflects *A. vulgaris*' intolerance of warm summer conditions on the inner continental shelf, and possibly a competitive disadvantage vis-a-vis *A. forbesi.* The shallower lower-depth limit toward the south is not easily explained. Hutchins' (1947) model predicts that eurythermal cold-water species limited in their southward distribution by the effects of warm temperatures on reproduction should emerge southward, since winter temperatures suitable for reproduction will occur in progressively shallower waters to the south. However, this cannot account for the observed patterns in *A. vulgaris*, because summer bottom temperatures at depths ≥ 100 m are generally between 10°C and 17°C, a favorable temperature range for *A. vulgaris.* Two factors that may affect the success of *A. vulgaris* at moderate depths are competition with *A. forbesi* and recruitment. The lower depth limit of *A. forbesi* becomes deeper over the same depth and latitudinal range in which *A. vulgaris* becomes shallower. Perhaps the relatively warm winter conditions, and warmer summer bottom temperatures south of 38°N favor *A. forbesi* over *A. vulgaris.* The recruitment of *A. forbesi* in this zone might also be favored over *A. vulgaris.* Southward-flowing long-shore currents along the Virginia/North Carolina coastal shelf are deflected northeasterward inshore of the northeasterward-flowing Gulf Stream (Ford and Miller, 1952). Thus, recruitment of *Asterias* spp.





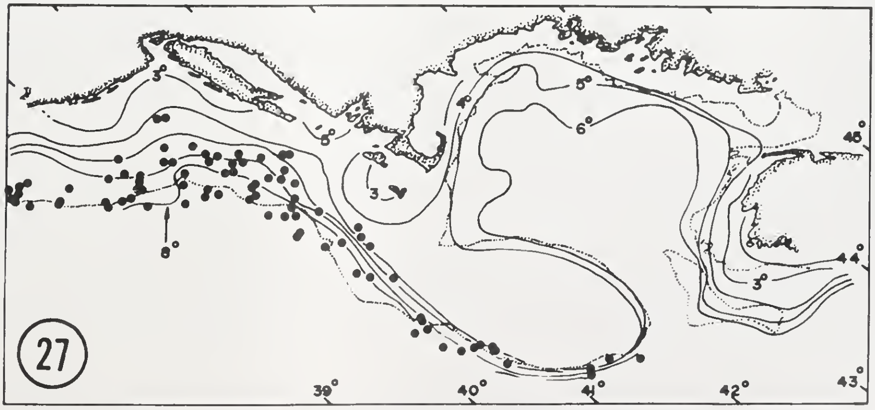


FIGURE 25. Distribution of all arctic-subarctic (open circles) and subarctic (solid circles) species in relation to September bottom isotherms, Gulf of Maine to Cape May (New Jersey). Compressed isotherms associated with Nantucket Shoals and Georges Bank constitute a sharp thermal barrier to the southward distribution of these species.

FIGURE 26. Distribution of all subarctic-boreal species (excluding *Henricia* spp.) in relation to September bottom isotherms, Gulf of Maine to Cape May (New Jersey). Note that subarctic-boreal species are not constrained in their distribution by bottom temperatures in the Gulf of Maine, and are thus relatively immune to the effects of the strong thermal barrier associated with Georges Bank and Nantucket Shoals.

FIGURE 27. Distribution of all boreal outer shelf species in relation to February bottom isotherms, Gulf of Maine to Cape May (New Jersey). Winter temperatures on the inner continental shelf and Georges Bank prevent the northward extension of these species.

on the outer continental shelf just north of Cape Hatteras probably depends on populations of larvae carried by these currents. Many more *A. forbesi* larvae probably are entrained by such currents than larvae of *A. vulgaris*, because of the relative scarcity of *A. vulgaris* on the inner continental shelf off North Carolina and Virginia.

Faunal groups of continental shelf seastars

The six faunal groups of seastars proposed above are based on similarities in depth and latitudinal distribution patterns, and similarities in temperature-distribution correlations. We suggest that these faunal groups represent more than random aggregations of animals with similar physiological requirements. They may also share similar paleobiogeographical patterns, such as similar origins in space, and presence in the Northwest Atlantic for comparable lengths of time. Direct evidence relating to the paleobiogeography of North Atlantic asteroids is lacking, so hypotheses based on this approach must depend, at least for the present, on analogies between faunal patterns in seastars and testaceous invertebrate taxa such as Mollusca and Foraminifera, for which fossil records already exist.

Franz and Merrill (1980a, b) suggested that warm-water mollusks with geographical ranges that extend north of Cape Hatteras (the "transhatteran" fauna) are derived from the Miocene fauna of the North Atlantic, and characteristically range southward to Florida, the Gulf of Mexico, and the Caribbean. Although *A. forbesi* does occur southward to the Florida Keys, and was reported by Verrill (1895) in the northern Gulf of Mexico, a review of distributional records in the collections of the United States National Museum suggests that it is rare south of the Cape Kennedy/Melbourne area. It is absent from museum collections from the Gulf of Mexico. Similarly, the records listed by Downey (1973) in her monograph of Caribbean and Gulf of Mexico starfish are all from the east coast of Florida north of latitude 28°N. These data indicate that while *A. forbesi* ranges south of the Cape Hatteras thermal barrier, its southern limits seem to be the eastern coast of Florida: it may occur rarely or not at all in the Gulf of Mexico. In this regard, its distribution pattern is different from those exhibited by most continental shelf transhatteran species of mollusks.

A. forbesi is closely related to *A. vulgaris* (Schopf and Murphy, 1973), *A. rubens*, and *A. amurensis* (Fisher, 1930), all species of the North Atlantic or the North Pacific. Worley and Franz (in preparation) hypothesize that these species share a common North Pacific ancestor and that this genus entered the North Atlantic via the Arctic in the late Miocene or Pliocene. If so, the transhatteran pattern of *A. forbesi* is not evidence of a Miocene Atlantic ancestry, but nevertheless reflects a relatively long period of adaptation to the marine climate of the Northwest Atlantic.

Franz and Merrill (1980b) postulate that the endemic boreal component of the molluscan shelf fauna comprises species either of Miocene Atlantic progenitors, or species of North Pacific origin, but with long evolutionary histories in the North Atlantic. The seastars of Group 3, the Northwest Atlantic outer shelf boreal species, would appear to fit the former pattern. The genera *Sclerasterias, Astropecten,* and *Porania* are predominantly warm-water cosmopolitan (Fisher, 1911, 1928). All of the species of this group are endemic to the boreal zone of the Northwest Atlantic, and all of the genera are characterized by a marked diversification south of Cape Hatteras. Thus, these genera probably have ancient (pre-miocene) Atlantic or Tethyan origins.

The species of Group 4, with geographical ranges including the boreal zone and extending into subarctic waters, and exhibiting boreal submergence, are similar to the amphiatlantic component of the boreal mollusks described by Franz and Merrill (1980a, b). The two seastars *Asterias vulgaris* and *Leptasterias tenera* are closely related to the Northeastern Atlantic congeners *A. rubens* and *L. mulleri*. All of these species are unquestionably of northern origin, with closest relatives in the subarctic Atlantic, the Arctic Ocean, Bering Sea, and boreal North Pacific. The progenitors of *L. tenera* and *A. vulgaris* probably entered the Atlantic via the Arctic (Nesis, 1961) in the Pliocene, and these species evolved separately in the Northeast and Northwest Atlantic during the Pliocene and Pleistocene.

Groups 5 and 6, the subarctic and arctic-subarctic species, are similar in being entirely amphiatlantic, and in their generally eurybathic depth ranges (Tables IV, V). Three of the species of Group 5 are not found in the Bering Sea or North Pacific, although *Hippasteria phrygiana* is closely related to the North Pacific *H. spinosa* (Nesis, 1961). According to Grainger (1966), *Ceramaster granularis* occurs in the Bering Sea while *Leptychaster arcticus* and *Pseudorchaster parelii* are found in the Bering Sea and the Northwest Pacific. All three species have discontinuous Atlantic-Pacific distributions, *i.e.*, they are present in the North Atlantic and North Pacific but absent from the Arctic Ocean. In a recent analysis of the Mollusca of the Western Beaufort Sea, Bernard (1979) indicated that 13 of 24 species of probable Atlantic origin do not extend into the Bering Sea; and that only 1 of 21 species of probable Pacific origin does not occur now in the Bering Sea. This implies that the Group 5 fauna may be of mixed origins, including genera with Atlantic origins (*Poraniomorpha, Psilaster*) and Pacific origins (*Hippasteria, Ceramaster, Leptychaster, Pseudarchaster*). The latter were probably distributed continuously when the Arctic was slightly warmer than at present (Mortensen, 1932; Ekman, 1953; Nesis, 1961), or evolved from progenitors that migrated from the North Pacific.

The Bering Strait may have opened and closed several times in the late Miocene, Pliocene, and Pleistocene (Hopkins, 1973). When the Strait was open, animals migrated between the North Pacific, Arctic, and Atlantic (Nesis, 1961). Thus, several waves of immigration of Pacific species into the Arctic and into the Atlantic (and vice versa) would have taken place. The earliest immigrants, because of their longer history in the North Atlantic, would be expected to show the greatest divergence from their North Pacific congeners. Most of the subarctic species of Group 5 exemplify this pattern, *i.e.* species which evolved in the Atlantic from early transarctic migrants. Eastern and western Atlantic species also would become differentiated during the Pliocene as populations became better adapted to the rather rapidly changing environments of the Northwest and Northeast Atlantic. The disappearance of connectant populations along the North Atlantic Arc, as glacial conditions developed in Greenland and Iceland in the Pliocene, probably facilitated speciation. Pairs of related species such as *Asterias forbesi* and *A. rubens*, and *Leptasterias tenera* and *L. mulleri*, may have differentiated in the North Atlantic by this process.

Species that now have continuous Arctic distribution patterns, but that extend their ranges into the North Atlantic and into the North Pacific, would be expected to exhibit less regional morphological differentiation, and to be less well adapted to the specific environment of the northwest Atlantic shelf. The arctic-subarctic species of Group 6 may represent this pattern.

If our hypotheses are valid, the origins of the continental shelf asteroid fauna of the Northwest Atlantic are complex. The fauna is composed of a mixture of groups of different origins in time, and various degrees of specific differentiation, probably resulting from sporadic transarctic migrations. We reached the same conclusions in our analysis of molluscan distribution patterns. However, one major discrepancy between mollusks and asteroids is apparent. While the shallow shelf and shore molluscan fauna between Cape Hatteras and Cape Cod is dominated by species of Miocene/Pliocene Atlantic origins (the transhatteran fauna), the diversity of seastars occupying this zone is extremely low. South of Cape Hatteras seastar diversity increases nearshore and on the continental shelf, an increase accounted for by additional endemic subtropical-tropical taxa. The absence of a transhatteran component of endemic, warm-water starfish—a faunal group very prominent in the mollusks—is unexplained. The answer may lie in the character of the Miocene shore environment of the East Coast of North America, dominated as it probably was by shallow embayments and great estuaries relatively non-conducive to asteroid diversification.

Faunal barriers

The distribution patterns of seastars re-emphasize an important point: for continental shelf species, Cape Hatteras and Cape Cod, *per se*, are not barriers. Thermal discontinuities associated with the confluence of surface currents, and influenced by the configurations of the continental shelf, are barriers to the distribution of some faunal groups. As noted earlier, the northward ranges of *Luidia clathrata, Astropecten articulatus*, and *Stephanasterias gracilis* are not directly affected by the geophysical presence of Cape Hatteras, but by the seasonal chilling of inshore waters north of Cape Hatteras under the influence of the coastal longshore currents. This is also true of the Cape Cod region. The major faunal barriers observed in this study are thermal, associated with the compression of bottom isotherms along the margins of Georges Bank and Nantucket Shoals. This is illustrated in Figures 25 and 26, which show the distribution of arctic-subarctic, subarctic, and subarctic-boreal species (excluding *Henricia*) superimposed on September bottom isotherms. Note that the range limits of both the arctic-subarctic and subarctic faunal groups are closely correlated with the compressed summer bottom isotherms associated with Nantucket Shoals and the northern slope of Georges Bank (as noted also for ostracods by Hazel, 1970, polychaetes by Kinner, 1977, and amphipods by Watling, 1979). However, the subarctic-boreal species are not affected by these isotherms and extend their ranges across Georges Bank and into the Gulf of Maine. The outer shelf boreal group (Fig. 27) is also limited by compressed isotherms; but in this case by winter isotherms associated with the southern slope of Georges Bank. Winter bottom temperatures on Georges Bank and on the northern slope (4°–6°C) inhibit the northward extension of these species.

ACKNOWLEDGMENTS

We gratefully acknowledge the unstinting assistance and cooperation of the personnel of the National Marine Fisheries Service at both Woods Hole and Sandy Hook. Dr. M. J. Cerame-Vivas identified all of the seastars from Cruise 60-7. We thank Dr. Ailsa Clark of the British Museum of Natural History, and Maureen Downey of the Echinoderm Division, United States National Museum, for their assistance in identifying certain specimens. Maureen Downey kindly confirmed all of our identifications. We also thank Dr. Gordon Hendler, Smithsonian Sorting Center, for constructive comments on this manuscript. This research was supported in part by a grant from the City University of New York (PSC-CUNY Grant No. 13315).

LITERATURE CITED

BERNARD, F. R. 1979. Bivalve mollusks of the Western Beaufort Sea. *Contrib. Sci. Nat. Hist. Mus. Los Angeles County* **313:** 1–80.

BOOLOOTIAN, R. A. 1966. Reproductive Physiology. Pp. 561–614 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience, New York.

BOWMAN, M. J., AND L. D. WUNDERLICH. 1977. Hydrographic properties. *MESA New York Bight Atlas* I, New York Sea Grant Inst., Albany, N. Y. 78 pp.

CLARK, H. L. 1904. The echinoderms of the Woods Hole Region. *Fish. Bull. U. S. Fish Wildl. Serv. 1902.* **22:** 545–576.

COE, W. R. 1912. Echinoderms of Connecticut. Bull. 19, *Conn. Geol. Nat. Hist. Surv.* Hartford, CT. 152 pp.

COLTON, J. B., AND R. R. STODDARD. 1973. Bottom-water temperatures on the continental shelf, Nova Scotia to New Jersey. *NOAA Tech. Rept. NMFS Circ-376.* 55 pp.

DOWNEY, M. E. 1973. Starfishes from the Caribbean and the Gulf of Mexico. *Smithsonian Contr. Zool.* **126:** 158 pp.

DUNBAR, M. J. 1951. Eastern Arctic waters. *Bull. Fish. Res. Bd. Can.,* **88.** 131 pp.

DUNBAR, M. J. 1953. Arctic and subarctic marine ecology: immediate problems. *Arctic* **6(2):** 75–90.

EKMAN, S. 1953. *Zoogeography of the Sea.* Sidgwick & Jackson, London. 417 pp.

FEDER, H. M., AND A. M. CHRISTENSEN. 1966. Aspects of asteroid biology. Pp. 88–127 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience, New York.

FISHER, W. K. 1911. Asteroidea of the North Pacific and adjacent waters. Pt. 1. Phanerozonia and Spinulosa. *Bull. U. S. Nat. Mus.* **76.** 419 pp.

FISHER, W. K. 1928. Asteroidea of the North Pacific and adjacent waters. Pt. 2. Forcipulata (part). *Bull. U. S. Nat. Mus.* **76.** 245 pp.

FISHER, W. K. 1930. Asteroidea of the North Pacific and adjacent waters. Pt. 3, Forcipulata, *Bull. U. S. Nat. Mus.* **76.** 356 pp.

FORD, W. L., AND A. R. MILLER. 1952. The surface layer of the Gulf Stream and adjacent waters. *J. Mar. Res.* **11:** 267–280.

FRANZ, D. R., AND A. S. MERRILL. 1980a. Molluscan distribution patterns on the continental shelf of the Northwest Atlantic. *Malacologia* **19(2):** 209–225.

FRANZ, D. R., AND A. S. MERRILL. 1980b. The origins and determinants of distribution of molluscan faunal groups on the shallow continental shelf of the Northwest Atlantic. *Malacologia* **19(2):** 227–248.

GALTSOFF, P. S., AND V. J. LOOSANOFF. 1939. Natural history and methods controlling the starfish (*Asterias forbesi* Desor). *Bull. U. S. Bur. Fish.* **49(31):** 75–132.

GRAINGER, E. H. 1964. Asteroidea of the "Blue Dolphin" expedition to Labrador. *Proc. U. S. Nat. Mus.* **114(3478):** 31–46.

GRAINGER, E. H. 1966. Sea Stars (Echinodermata:Asteroidea) of arctic North America. *Bull. Fish Res. Bd. Com.* **152.** 70 pp.

GRAY, I. E., M. DOWNEY, AND M. J. CERAME-VIVAS. 1968. Sea stars of North Carolina. *U. S. Fish. Wildl. Serv. Fish. Bull.* **67(1):** 127–163.

HALL, C. A. 1964. Shallow-water marine climates and molluscan provinces. *Ecol.* **45(2):** 226–234.

HANCOCK, D. A. 1955. The feeding behavior of starfish on Essex oyster beds. *J. Mar. Biol. Assoc. U. K.* **34:** 313–331.

HAYNES, E. B., AND R. L. WIGLEY. 1969. Biology of the northern shrimp, *Pandalus borealis*, in the Gulf of Maine. *Trans. Am. Fish Soc.* **98(1):** 60–76.

HAZEL, J. E. 1970. Atlantic continental shelf and slope of the United States—Ostracode Zoogeography in the southern Nova Scotian and northern Virginian faunal provinces. *U. S. Geol. Surv. Prof. Pap.* **S29-E.** 21 pp.

HOPKINS, D. M. 1973. Sea level history in Beringia during the past 250,000 years. *Quat. Res.* **3:** 520–540.

HUTCHINS, L. W. 1947. The basis for temperature zonation in geographical distribution. *Ecol. Monogr.* **17:** 325–335.

KINNER, P. C. 1977. The distribution and ecology of errantiate polychaetes on the continental shelf from Cape Cod to Cape Hatteras. *M. S. Thesis, Univ. Delaware.* 122 pp.

Krebs, C. J. 1978. *Ecology: The experimental analysis of distribution and abundance.* Harper & Row, New York. 678 pp.

Loosanoff, V. L. 1961. Biology and methods of controlling the starfish, *Asterias forbesi* (Desor). *U. S. Fish Wildlf. Bur. Fish. Leaflet* **520.** 11 pp.

MacKenzie, C. L. 1969. Feeding rates of starfish, *Asterias forbesi* Desor at controlled temperatures and during different seasons of the year. *U. S. Fish. Wildl. Serv.* **68(1)**: 67–72.

Mileikovsky, S. A. 1970. Seasonal and daily dynamics in pelagic larvae of marine and shelf bottom invertebrates in nearshore waters of Kandalaksha Bay (White Sea). *Mar. Biol.* **5**: 180–194.

Mileikovsky, S. A. 1971. Types of larval development in marine bottom invertebrates, their distribution and ecological significance: a re-evaluation. *Mar. Biol.* **10(3)**: 193–213.

Mortensen, T. 1927. *Handbook of the echinoderms of the British Isles.* Oxford Univ. Press, London. 471 pp.

Mortensen, T. 1932. Echinoderms. The Godthaab Expedition 1978. *Medd. om Grønland.* **79(2)**: 62 pp.

Needler, A. W. H. 1941. Oyster farming in Canada. *Bull. Fish. Res. Bd. Can.* **60**: 83 pp.

Nesis, K. N. 1961. The routes and periods of formation, of the interrupted area of distribution of amphiboreal species of marine bottom animals. *Okeanologiya* **1(5)**: 894–903.

Sanders, H. L. 1968. Marine benthic diversity: a comparative study. *Am. Nat.* **102**: 243–282.

Sanders, H. L. 1969. Benthic marine diversity and the stability-time hypothesis. *Brookhaven Symposium on Biology* **22**: 71–81.

Schopf, T. J. M., and L. S. Murphy. 1973. Protein polymorphism of the hybridizing seastars *Asterias forbesi* and *Asterias vulgaris* and implications for their evolution. *Biol. Bull.* **145**: 587–597.

Smith, G. F. M. 1940. Factors limiting distribution and size in starfish. *J. Fish Res. Bd. Can.* **5**: 84–103.

Ursin, E. 1960. A quantitative investigation of the echinoderm fauna of the central North Sea. *Medd. Danm. Fisk-Havunders* **2**: 1–104.

Verrill, A. E. 1878. Notice of recent additions to the marine fauna of the eastern coast of North America. *Am. J. Sci.* **16**: 207, 231.

Verrill, A. E. 1894. Descriptions of new species of starfish and ophiurans. *Proc. U.S. Nat. Mus.* **17**: 245–297.

Verrill, A. E. 1895. Distribution of the echinoderms of Northeastern America. *Am. J. Sci. Ser.* **3, XLII (Arts 13, 19)**: 127–141, 199–212.

Walford, L. A., and R. I. Wicklund. 1968. Monthly sea temperature structure from the Florida Keys to Cape Cod. *Ser. Atlas Mar. Environ. Am. Geogr. Soc.* **(15)**: 27 pp.

Watling, L. 1979. Zoogeographic affinities of northeastern North American gammaridean Amphipoda. *Bull. Biol. Soc. Wash. (1979)* **3**: 256–282.

Wigley, R. L., R. B. Theroux, and H. E. Murray. 1975. Deep-sea red crab, *Geryon quinquidens*, survey off Northeastern United States. *Marine Fish. Rev.* **37(8)**: 22 pp.

Zinn, D. J. 1937. The growth and development of starfish in Narragansett Bay in relation to temperature and food supply. *M. S. Thesis, Univ. Rhode Island*, 38 pp.

Reference: *Biol. Bull.* **160:** 419–430. (June, 1981)

ORIENTED LIGHT REACTIONS OF THE ARROW WORM *SAGITTA CRASSA* TOKIOKA

TAICHIRO GOTO AND M. YOSHIDA

*Ushimado Marine Laboratory, Kashino, Ushimado, Okayama 701-43 Japan**

ABSTRACT

The arrow worm *Sagitta crassa* shows positive phototaxis in a horizontal light beam by repeatedly swimming briefly upward with the body axis inclined toward the illuminated side, and then by passively sinking. Phototactic behavior is less evident in vertical beams. When a horizontal light beam is reduced after a period of illumination, the arrow worms have a fast goal seeking response, steering themselves toward the reduced light source in less than 0.3 s and swimming straight to this target at rates greater than 14 cm/s. When two light targets are presented immediately after a reduction in light intensity, the arrow worms choose the brighter one, indicating that the response may be a type of telotaxis. The two kinds of phototactic swimming may be explained by slow taxis by changes in orientation, and target-aiming behavior by two sequential processes: an arousal increase followed by an activity release.

INTRODUCTION

Chaetognaths, commonly called arrow worms from their elegant appearance and swimming behavior, are a small, phylogenetically isolated group of marine planktonic predators. Next to copepods, they are the commonest component of the zooplankton. Consequently, they are important in pelagic ecosystems. Apart from field studies on diurnal vertical migration patterns (Michael, 1911; Russell, 1927, 1931; Murakami, 1959; Pearre, 1973; Nagasawa and Marumo, 1975; Kotori, 1976; Hirota, 1979; Goto, 1980), little is known about physical factors that control their pattern of swimming behavior. Esterly (1919) and Pearre (1973), working with *Sagitta bipunctata* and *S. elegans* respectively, show that both these species are strongly photopositive in a horizontal light beam, negatively geotactic in a vertical tube in darkness, and positively geotactic in a bright light. We present below our detailed analyses of light oriented movements (phototaxis) in *Sagitta crassa*, including a new type of a target-aiming (telotactic) quick swimming behavior.

MATERIALS AND METHODS

Arrow worms, *Sagitta crassa*, were collected at night by slowly towing horizontally XX 13 gauge net (mesh size 95 μm square). To avoid unduly damaging the worms, a small collecting bottle, 13 cm in diameter and height, with a leak pipe at the center, was held at the cod end of the net. Mature worms at Stage III of Pierce's classification (1951) captured in this manner were maintained in a seawater-filled trough (23 cm in diameter and 10 cm in height) at 17°C under a cycle of 12 h light:12 h darkness. Worms could be kept healthy for at least 3 weeks by keeping less than 10 in each trough, feeding them with freshly hatched *Artemia salina* nauplii every 3 days, and changing the seawater at each feeding.

Received 13 January 1981; accepted 13 March 1981.

Abbreviations: $-\Delta I$, light intensity reduction; L_1, L_2, target lights; CL, conditioning light.

* Formerly Tamano Marine Laboratory.

Tungsten filament lamps (100 V, 150 W) were used as light sources. The light beam which passed through infrared absorbing filters (Toshiba IRA 25 S) and neutral density filters (Toshiba), was made approximately parallel by a lens system. Details of experimental procedures are described below.

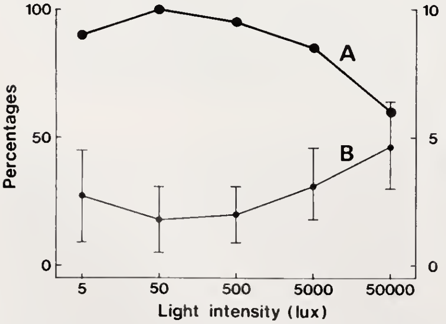
RESULTS

Slow tactic behavior

A shallow transparent trough (20 × 4 × 4 cm) was used. At the start of each experiment, a worm adapted to darkness for longer than 10 min was placed in a 5 cm compartment behind a partition that was 15 cm from the wall of the trough and that faced a horizontally directed light source. Positively phototactic swimming behaviors were examined by removing the partition and simultaneously opening the shutter of the light source, and counting numbers of worms that reached the illuminated side within 10 min. Most worms responded positively to moderate intensities of light (Fig. 1, curve A) and remained at the illuminated region of the trough, moving back and forth to a small extent. This effect was less pronounced with the brightest light.

Phototactic worms did not swim directly toward the light source. Instead, while responding positively (Fig. 1, curve B), they shifted their position in a series of upward swimming and downward maneuvers, rather like the "hop and sink" behavior described for other plankton (Fig. 2). Close examination of the path followed shows that the body axis of the upward swimming worms tended to be inclined toward the light source.

These inclination angles were measured by photographing with strobe flashes every 15 s for 2 min while worms were kept in darkness (control) or were exposed to a horizontal light beam of various intensities. Results (Fig. 3) show that in a horizontal light beam of 500 lux (B), about three times as many arrow worms incline toward the light than away from it (60.3 + 7.1% vs. 18.4 + 3.5%). Such preferred inclination will result in progressive movement toward the light, since arrow worms do not swim backward. The frequencies between inclinations toward and away from light appeared to differ in darkness (35.6% vs. 43.1%) as well as



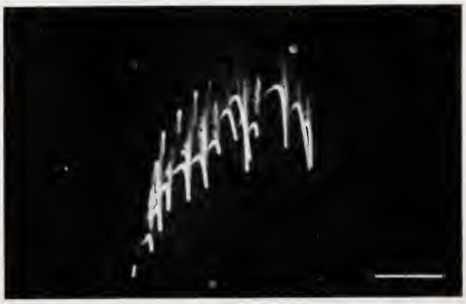


FIGURE 1. (Left) Phototaxis in a horizontal light beam. A: Percentages of positively photatactic worms (n = 20). B: Time required for the positively responded worms to reach the illuminated end. Vertical bars: Standard deviation.

FIGURE 2. (Right) Photograph taken by 30 s continuous exposure, showing a typical phototactic trail of an arrow worm in a thin tank (8 × 1.5 cm, 6 cm deep). Light (5000 lux) entering from the right. Scale: 1 cm.

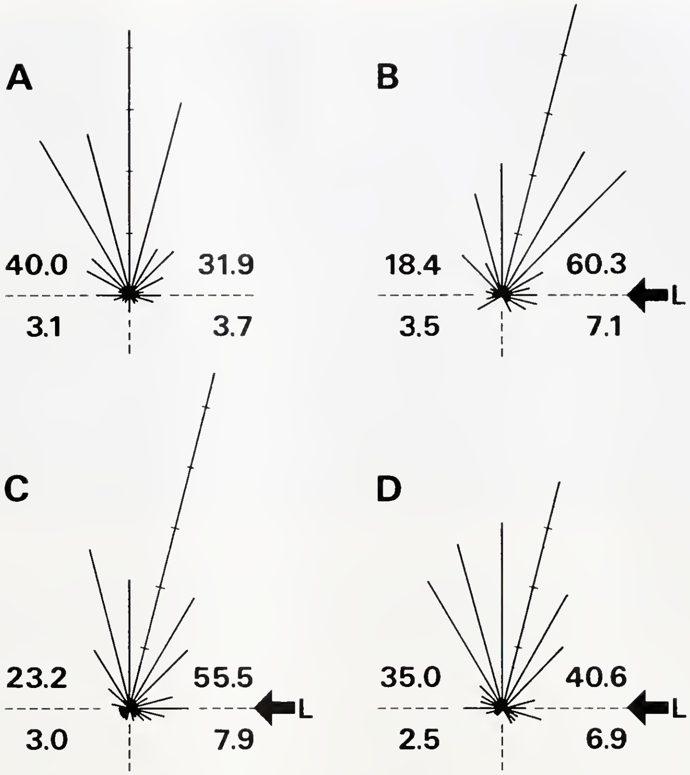


FIGURE 3. Inclination pattern of the body axis of worms measured at 15-degree intervals in darkness (A: Dark control, n = 163) and in a horizontal light beam (B: 500 lux, n = 141. C: 5000 lux, n = 164. D: 50,000 lux, n = 160). Lengths of each radiating line show the percentages of worms inclined in that direction (calibrations on the longest line show 5% intervals). Since upright (90°) and upside down (270°) orientations are irrelevant to the light oriented responses, the number of worms which took such orientations were summed separately. Numbers in each quadrant are total percentages in the respective direction.

in the bright light (47.5% vs. 37.5%). But the difference was insignificant ($0.3 < P < 0.5$ and $0.2 < P < 0.3$, respectively), indicating random movements.

If movement to the end of the experimental vessel illuminated by a horizontal beam is achieved by repeated inclined vertical movements, a vertical beam will not be as effective in attracting *Sagitta* as it has been reported to be in directly phototactic planktonic crustaceans. This was found to be true. Thus, few worms reached the illuminated water surface or bottom of a deep container (4 × 4 cm, 40 cm high) in downward- or upward-directed beams (Fig. 4A, curves a and b, respectively). Those animals presumably attracted to these points did not stay but soon swam away, though some animals remained in the middle and lower sections with upward directed beams (white bars in Fig. 4C). On the other hand, the fact that most of the worms were found in the upper region of the container after a dark period (initial distribution pattern; black bars in Fig. 4B, C) indicates that the worms are basically negatively geotactic. The increase in numbers in the middle and lower sections with brighter downward directed beams (white bars in Fig. 4B) indicates that light changed the sign of geotaxis.

Quick target-aiming behavior

While maintaining worms with an overhead light, we noticed that when the aquarium was shifted, a majority of the worms swam briskly straight toward the lamp side. Similar rapid responses were seen when the intensity of a horizontal light beam was suddenly reduced. Trails photographed by a continuous exposure (*e.g.*, Fig. 5) showed that this swimming pattern was different from that of the ordinary hop and sink phototactic behavior (Fig. 2). The response proceeded in two steps: First, the worms oriented themselves toward the reduced light source. Second, they swam straight toward it.

Cine camera recordings (36 frames per s) showed that the orientation was completed within 0.3 s and that the swimming speed was more than 14 cm per s. The rapid swimming is not achieved by an initial single stroke of the tail. Figure 5 shows brighter profiles (representing slow speed movements) at intervals along the more or less straight, faint (high speed) trails, indicating repeated successive bursts of rapid swimming. Other photographs show that swimming speed increased during the target-aiming response.

Effects of light-intensity reduction. We studied the effects of different amounts of light intensity reduction ($-\Delta I$) in evoking rapid swimming. In this and all the following experiments, experimental animals were selected on the basis of their ability to respond to 90% reduction of a horizontal light intensity of about 1000 lux, after 3–5 min illumination with this light. Such worms were assumed to be healthy. (Damage or deterioration might have occurred during collection and maintenance.) Usually about 30% of the worms collected met this behavioral criterion.

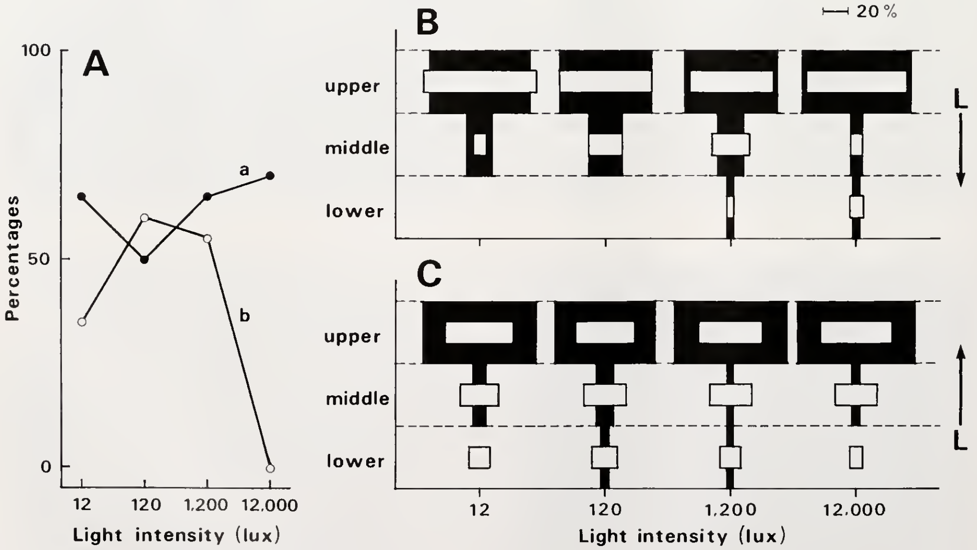


FIGURE 4. Phototaxis in vertical light beams. A: Percentages of worms (n = 20) which reached the water surface at least once with a downward directed beam, curve a, or the bottom of the tank with an upward directed beam, curve b. B and C: Vertical distribution patterns at the beginning (initial, black bars) and end (final, white bars) of a 10 min illumination period with either downward (B) or upward (C) directed beams after dark adaptation for 10 min. "Upper," "lower," and "middle" on the left designate, respectively, the upper one tenth (4 cm), lower one tenth (4 cm), and middle section (32 cm) between the two of a deep container.

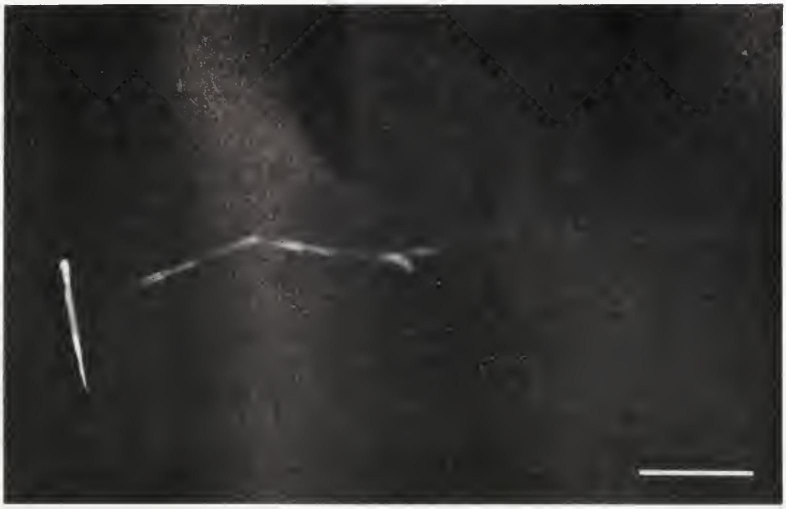


FIGURE 5. Photograph taken by a 3 s continuous exposure, showing an arrow worm's pattern of target-aiming swimming. Light (50,000 lux) directed from the right was reduced by 90%. Note that the worm, which had been inclined toward the left, changed its body axis to the right almost instantaneously: No trail linking the left-oriented posture to the right appears on the photograph, indicating a very quick change sequence. Scale: 1 cm.

In each experiment, a light-adapted worm was placed in a trough 20 cm long, 4 cm wide, and 4 cm deep, which was illuminated from both ends, one end at 800 lux and the other varying from 160–2400 lux. After 5–10 min, the fixed-intensity light was turned off and whether the worm oriented and swam toward the remaining light was recorded (Fig. 6). The larger $-\Delta I$, the more worms swam toward the light. The mechanisms involved in the response's two steps (steer and swim) differed in sensitivity, so that a majority only steered themselves upon small $-\Delta I$'s, whereas all the animals went on to the second step (swim) in response to the largest $-\Delta I$.

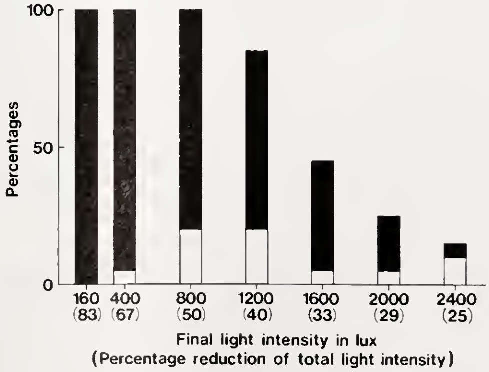
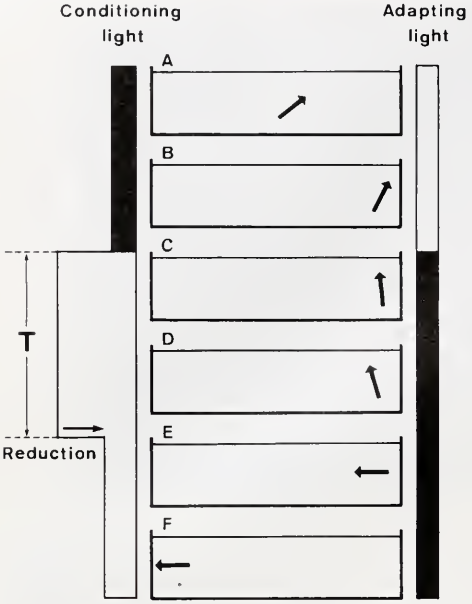


FIGURE 6. Target-aiming response following intensity reduction by extinguishing a light of 800 lux from one end, showing percentages of worms (n = 20) that steered (white bars) or steered and swam (black bars) toward the remaining light at the opposite end of the trough (abscissa). The reduction in intensity is indicated in brackets along the abscissa.



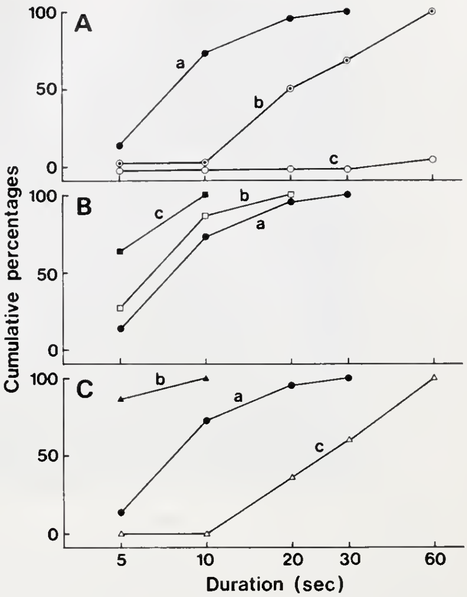


FIGURE 7. (Left) Schematic representation of the sequence of photic manipulations for testing rapid target-aiming behaviors. Arrows indicate arrow worms. T: Duration of illumination with the conditioning light. A: Illumination with an adapting light for 5–10 min. B: The worm reaches the illuminated end. C: Turning on of a conditioning light and off of the adapting light. D: The worm is illuminated by the conditioning light for a period of T. E: Orientation of the worm toward the conditioning light when it is reduced (arrow to the right). F: Quick swimming toward the reduced light source.

FIGURE 8. (Right) Effects of photic conditions on the critical period, the shortest duration of illumination with the conditioning light necessary to induce the target-aiming swimming (abscissa). The ordinate shows cumulative percentages of worms (n = 22) that responded with a critical period shorter than that on abscissa. Curves *a* are the same in all three figures (A, B, C). Photic conditions that varied are the degree of reduction in A (*a*, 90%; *b*, 70%; *c*, 50%), the intensity of the conditioning light in B (*a*, 600 lux; *b*, 180 lux; *c*, 60 lux) and the intensity of the adapting light in C (*a*, 12 lux; *b*, 120 lux; *c*, 0 lux).

Effects on response threshold of adapting and conditioning lights. To elucidate physical factors necessary to induce these responses, we manipulated light conditions as follows (Fig. 7): A dark adapted worm was put in the trough and an adapting light on one end was turned on for 5–10 min (A). Within this period, the worm reached the illuminated end by its positive phototaxis (B). Another conditioning light on the opposite side was then turned on, while the adapting light was simultaneously turned off (C). After a certain period of illumination (T) the intensity of the conditioning light was reduced suddenly by interposing neutral density filters (E).

Thus, photic conditions that might affect responses are the intensities of the adapting and conditioning lights and −ΔI's. The effectiveness of each photic condition may be expressed by the shortest illumination time (to be called a critical period) necessary to induce the responses. In the experiments, critical periods were determined by stepwise increases (5, 10, 20, 30, 60 s, or longer) until the worm responded to −ΔI. When no response was induced with a given period of illumination, each worm was dark adapted for 5 min before starting the next step with 5–10 min illumination by the adapting light.

Experiments were performed by varying one of the three photic factors while keeping the other two constant (Fig. 8). When $-\Delta I$ was varied (Fig. 8A), only one of 22 worms responded to 50% reduction within a critical period as long as 60 s (curve c). A 70% reduction (curve b) made the critical period shorter than 60 s in all the worms tested, and 20 s was long enough to induce responses in half the worms tested. A 90% reduction made the critical period mostly shorter than 10 s. Thus, the larger the $-\Delta I$, the shorter is the required duration of the conditioning illumination. Conversely, longer conditioning exposures lower the threshold for $-\Delta I$. Indeed, when the worms were exposed to 1600 lux for more than 30 min, a 25% reduction induced rapid swimming behavior.

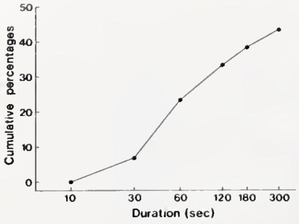
Under varying intensities of the conditioning light with 90% $-\Delta I$ (Fig. 8B), the critical periods were about the same under 180 lux and 600 lux (curves a and b) but became shorter under 60 lux (curve c). Figure 8C shows the results of varying the adapting light intensities with a 90% reduction of a 600 lux conditioning light. With no adapting light, the critical period became significantly longer (curve c). When, however, the intensities of the adapting light were equal to (60 lux; not shown in the figure) or higher than (120 lux) the reduced level of the conditioning light (60 lux), a shorter illumination (less than 10 s) with the conditioning light induced the response in all the worms tested (curve b). Thus, the adapting light that was used to lead worms to one side of the experimental vessel also increased the readiness of the worms to respond to an intensity reduction.

However, $-\Delta I$ was not the only stimulus that induced such rapid target-aiming responses. A single mechanical shock to the container—knocking, shifting, etc.—was sometimes effective. As shown in Figure 9, more animals orient toward the light source in response to single mechanical shocks as illumination is prolonged. This implies that during light adaptation, worms become ready (arousal) to orient themselves toward the light when stimulated by a releasing signal, which may be a light intensity reduction, a mechanical shock, or some other stimulus. Following orientation, responding worms would next swim directly to the target. Indeed, totally shaded worms swam around briskly but in random directions, as they had no obvious goal. The initial changes of the body axis toward the light, which occurred as a first step in Figure 5, must indicate that the worm can locate the target visually.

Effects of two stimuli. To determine what worms would do given two targets, the following experiments were performed, using three light sources (Fig. 10A, B): Worms put in the trough were illuminated by the conditioning light (1500 lux) for 10 min. Target lights (I_1, I_2) were then turned on and the conditioning light (CL) simultaneously extinguished. The intensity of I_2 was varied in a range between 18 and 600 lux while keeping I_1 at 120 lux, and the number of times the worms chose each target was determined. Most worms that responded to $-\Delta I$ aimed at target I_2 when I_2 was brighter than 150 lux (Fig. 10C). Since all the worms tested moved toward one of the two targets, a plot of percentages of worms that moved toward the target I_1 would be a mirror image of the curve in Figure 10C.

Some of the worms chose a dimmer target when I_2 was in the range 60–150 lux (25% in 60 lux and 15% in 150 lux). However, as the 50% level of the curve passes near the 120 lux line, where the intensities of the two targets were equal, the worms apparently made a 50–50 or random choice in this range. Therefore, worms aroused to be ready to respond will steer toward the brighter of two targets when released by an intensity reduction.

Effects of vertical light sources. As was done with horizontal lights, responses



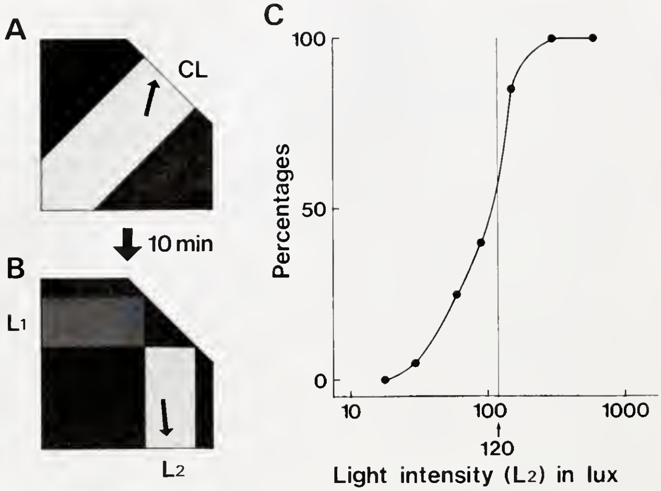


FIGURE 9. (Left) Effects of a mechanical shock on the orientation reaction of the body axis toward the light source (400 lux). Ordinate: Cumulative percentages of worms (n = 60) which responded with a critical period shorter than that shown on the abscissa. The mechanical shock was given by a solenoid which was triggered by a condenser discharge and which shifted the trough by 0.8 mm in 15 msec at an initial rate of 6 cm/s.

FIGURE 10. (Right) Choice of two targets. A and B show a trough and lighting conditions. CL: Conditioning light (1500 lux). L_1 (120 lux) and L_2 (varied: 18–600 lux) served as targets. Lighter stipplings indicate brighter light. Black arrow indicates an arrow worm. In C, percentages of worms (n = 20) which moved toward L_2 are plotted on the ordinate. Abscissa: Intensity of L_2 relative to L_1, whose (fixed) level is shown by an arrow.

in vertical light beams directed from above or below were examined by determining the critical period necessary to induce responses to various $-\Delta I$'s (Fig. 11). The procedure differed slightly, however. Single worms in each experiment were placed in the deep container under a dim red light (longer than 600 nm in wavelength). An adapting light (Fig. 7) was not used: Test illuminations began after 5 min dark adaptation.

As shown in Figure 11C, when the downward directed light beams of 3000 lux were reduced to 300 lux, critical periods were extremely long (curve d). Large $-\Delta I$'s, down to 30 lux, shortened the critical period markedly (curve c); and with even deeper shadings, to 3 and 0 lux (curves b and a, respectively), 30 s illumination induced responses in most worms. However, those upward movements may have been due to target-aiming or to negative geotaxis in darkness following elevated kinetic activity induced by light-intensity reduction.

Reduction of light directed from below was more effective in inducing rapid downward swimming. When the light was reduced to 300 or 30 lux, all the worms responded with critical periods shorter than 2 min (curves c and d in Fig. 11B). If, however, the beam intensity was reduced to 0–3 lux, a considerable number of worms swam upward (curves a′ and b′ in A), possibly due to geo-negativity in darkness.

DISCUSSION

Diurnal vertical migrations of arrow worms in the sea, rising at dusk and sinking at dawn, have been noted frequently (Michael, 1911; Russell, 1927, 1931; Mu-

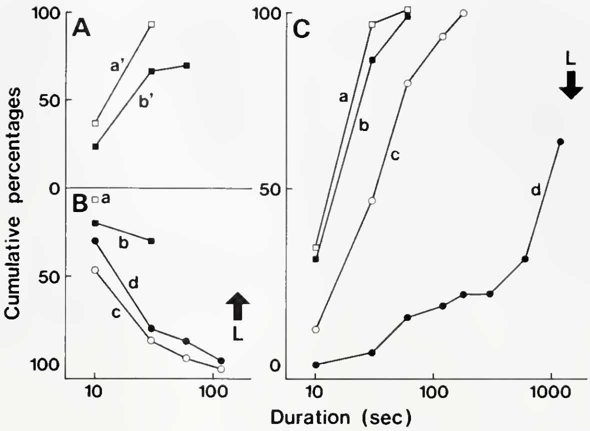


FIGURE 11. Effects of reduction in intensity of upward (A and B) and downward (C) directed light beams of 3000 lux. The reduced levels: 0 lux for curves *a* and *a'*, 3 lux for curves *b* and b', 30 lux for curves *c* and 300 lux for curves *d*. Ordinates: Cumulative percentages of worms (n = 30) that responded with critical periods shorter than those shown on abscissae. A and C show the percentages of worms that moved upward. B shows percentages of downward moving worms, plotted in such a way that downward directed lines indicate higher percentages of downward movements.

rakami, 1959; Pearre, 1973; Nagasawa and Marumo, 1975; Kotori, 1976; Hirota, 1979; Goto, 1980). Light has been considered a major factor controlling these movements. However, most work has been done in the field and little attention has been paid to behavior or physiology of individual worms in relation to environmental conditions. In addition to confirming the findings of Esterly (1919) and Pearre (1973) that worms are basically negatively geotactic, coming to the water surface when placed in darkness (Fig. 4), and that a horizontal light beam of moderate intensities makes worms gather on an illuminated side (Fig. 1), we have presented several new facts regarding light-oriented movements of arrow worms, *Sagitta crassa* (summary diagram in Fig. 12).

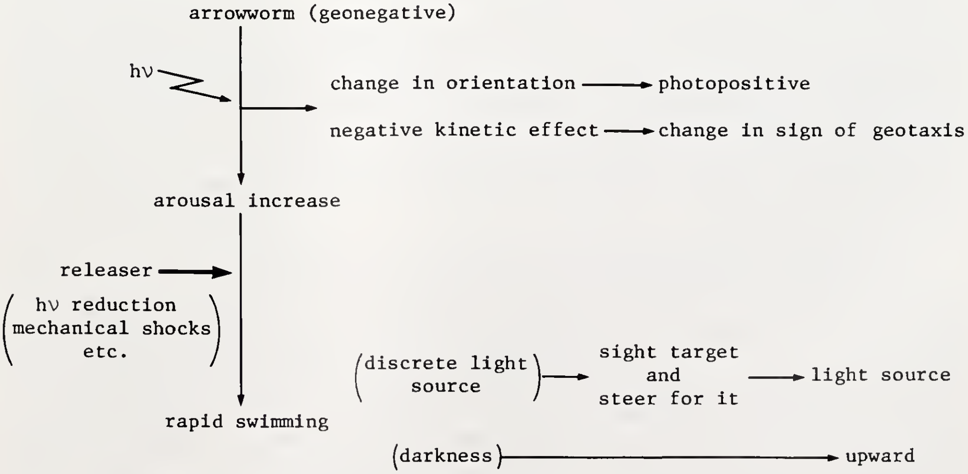

arrowworm (geonegative)
hν
change in orientation
photopositive
negative kinetic effect
change in sign of geotaxis
arousal increase
releaser
(hν reduction
mechanical shocks
etc.)
(discrete light source)
sight target and steer for it
light source
rapid swimming
(darkness)
upward

FIGURE 12. Summary diagram of the results.

When illuminated from the side, worms are rather slow to reach the illuminated end of a trough, even at the optimum light intensities to which all the worms respond positively (Fig. 1). Paths followed during this phototaxis show that the body axis of worms does not point directly toward the light source (Fig. 2). Instead, the body axis is significantly inclined toward light of moderate intensities, whereas in darkness and with brighter directed light, inclination toward one azimuth or another is more or less random (Fig. 3). Hence *Sagitta* shifts its position by repeated upward swimming movements with the body axis inclined toward the light source, interspersed with passive downward sinking.

The worms' more or less upright posture in darkness implies that they possess either a gravity sense or a gradient in specific gravity along the body axis, being heavier posteriorly. The latter possibility is not plausible, because when the worm is cut crosswise into three pieces, the head region sinks most rapidly. Also, anesthetized worms never sink with heads pointing upward. Since chaetognaths lack statocysts (Bullock, 1965), the gravity perception presumably is due to some other mechanism.

To induce light-oriented inclinations of the body axis, on the other hand, the effector system involved must be affected by gravity as well as information from light reception. Anterior, posterior, and tail fins, the bristles and cilia distributed over the body surface (Bone and Pulsford, 1978), and the ciliary loop on the dorsal side of the anterior region (Horridge and Boulton, 1967) are all possible candidates for the effector system. Other explanations, such as uneven contractions of the body wall by direct action of light, also are possible. It can be concluded that the light-oriented gathering is not a simple phototaxis or photokinesis, but is mediated by both gravity and light sensing mechanisms.

The quick target-aiming response induced by reduced light intensity (Figs. 6, 8, and 11), mechanical shocks (Fig. 9), etc., is an entirely new type of behavior in the laboratory. These two-step responses require a prior period of illumination during which the worms apparently do nothing, but become ready to respond to releasing stimuli (Fig. 12). On arrival of such a stimulus, the worms become kinetically active, and if those activated worms sight a target, they will steer toward it and start rapid swimming. In total darkness, they will swim upward due to their inherent negative geotaxis (see Figs. 4, 11, and 12).

The fact that worms presented with two targets do not swim along a resultant path of the two beams, but choose the brighter target (Fig. 10), may indicate that the behavior is a kind of telotaxis. Arrow worms possess a pair of pigmented ocelli. The pigment cell in each ocellus enables the receptive endings of sensory cells to differentiate incident light coming from various directions (Hesse, 1902; Burfield, 1927; Eakin and Westfall, 1964; Ducret, 1978; Goto, 1980). The existence of such a structure lends support to the notion of telotaxis, although, because of the delicate structure of the worm, direct proof by unilateral removal of the paired ocelli has not yet been obtained.

Chaetognaths are predators. They have been assumed to detect prey by sensing vibrations (Horridge and Boulton, 1967; Newbury, 1972). However, since the target-aiming swimming analyzed by us is quite accurate and rapid (14 cm per s), arrow worms may locate their prey visually and approach it telotactically when the prey (seen as a shadow) is swimming well outside the detectable range of vibration sensitivity, which is only 1–3 mm. Field observations of *Sagitta* feeding on larval herring (Lebour, 1923) suggest that our laboratory observations may have direct relevance to the predatory behavior in these animals.

However, *Sagitta*'s ability to catch prey at night and its simple lensless eyes lead one to be suspicious of such a notion. It is perhaps more likely that the observed behavior is an escape reaction, although in such responses, which follow shading in many planktonic crustaceans, movement is mostly away from the shadow (*e.g.*, Forward, 1974, 1976, 1977; Forward and Costlow, 1974). *Sagitta crassa* moves toward the shadow when only one light source is used. When more than one light source is used, the worms swim toward the brighter one. Such an ability would lead the worm in the sea to dodge away from approaching predators. Release of such reactions by mechanical shocks supports this interpretation. However, these speculations are based on experimental results obtained under laboratory conditions. The true biological significance of this unique swimming activity has yet to be directly demonstrated.

ACKNOWLEDGMENTS

We thank Professor R. Hirota, Kumamoto University, for invaluable instructions for maintaining arrow worms; Professor G. O. Mackie, University of Victoria, for revision of the original manuscript; Professor K. S. Iwata, Niimi Junior College, for the use of his cine camera; Professor T. Yamaguchi, Dr. M. Yamamoto, and Mr. K. Ohtsu, Okayama University, for helpful suggestions; and Mr. M. Isozaki and Mr. W. Godo for technical assistance. This work was supported in part by grants in aid from the Ministry of Education to M. Yoshida.

LITERATURE CITED

BONE, Q., AND A. PULSFORD. 1978. The arrangement of ciliated sensory cells in *Spadella* (Chaetognaths). *J. Mar. Biol. Assoc. U. K.* **58:** 565–570.

BULLOCK, T. H. 1965. Chaetognatha. Pp 1560–1564 in T. H. Bullock and G. A. Horridge, Eds., *Structure and function in the nervous systems of invertebrates. Vol. II.* W. H. Freeman and Co., San Francisco and London.

BURFIELD, S. T. 1927. Sagitta. *Liverpool Mar. Biol. Comm. Mem.* **28:** 1–104.

DUCRET, F. 1978. Particularités structurales du système optique chez deux Chaetognathes (*Sagitta tasmanica* et *Eukrohnia hamata*) et incidences phylogenetiques. *Zoomorphologie* **91:** 201–215.

EAKIN, R. M., AND J. A. WESTFALL. 1964. Fine structures of the eye of a chaetognath. *J. Cell Biol.* **21:** 115–132.

ESTERLY, C. O. 1919. Reactions of various plankton animals with reference to their diurnal migrations. *Univ. Calif. Publi. Zool.* **19:** 1–83.

FORWARD, R. B., JR. 1974. Negative phototaxis in crustacean larvae: Possible functional significance. *J. Exp. Mar. Biol. Ecol.* **16:** 11–17.

FORWARD, R. B., JR. 1976. A shadow response in a larval crustacean. *Biol. Bull.* **151:** 126–140.

FORWARD, R. B., JR. 1977. Occurrence of a shadow response among brachyuran larvae. *Mar. Biol.* **39:** 331–341.

FORWARD, R. B. JR., AND J. D. COSTLOW, JR. 1974. The ontogeny of phototaxis by larvae of the crab *Rhithropanopeus harrisii. Mar. Biol.* **26:** 27–33.

GOTO, T. 1980. Vertical migration and photoresponses in *Sagitta crassa. Masters thesis, University of Okayama, Japan.* 18 pp.

HESSE, R. 1902. Untersuchungen über die Organe der Lichtempfindung bei niederen Thieren. III. Weitere Thatsachen. Allgemeines. *Z. Wissensch. Zool.* **72:** 565–656.

HIROTA, R. 1979. Diurnal migrations and patch formations in macroplankton in the inland sea. *Marine Science Monthly, Tokyo* **11:** 627–631 (in Japanese).

HORRIDGE, G. A., AND P. S. BOULTON. 1967. Prey detection by Chaetognatha via a vibration sense. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **168:** 413–419.

KOTORI, M. 1976. The biology of Chaetognatha in the Bering Sea and the northern North Pacific Ocean, with emphasis on *Sagitta elegans. Mem. Fac. Fish. Hokkaido Univ.* **23:** 95–183.

LEBOUR, M. V. 1923. The food of plankton organisms. II. *J. Mar. Biol. Assoc. U. K.* **13:** 70–92.

MICHAEL, E. L. 1911. Classification and vertical distribution of the Chaetognatha of the San Diego region. *Univ. Calif. Publi. Zool.* **8:** 21–186.

MURAKAMI, A. 1959. Marine biological study on the planktonic chaetognaths in the Seto Inland Sea.

Bull. Naikai Regional Fisheries Research Laboratory **12:** 1–69 (in Japanese with English abstract).

NAGASAWA, S., AND R. MARUMO. 1975. Distribution of a chaetognath, *Sagitta nagae* Alvariño in Suruga Bay, central Japan with notes on the zooplankton biomass. *Bull. Plankton Soc. Japan* **21:** 23–40.

NEWBURY, T. K. 1972. Vibration perception by chaetognaths. *Nature* **236:** 459–460.

PEARRE, S., JR. 1973. Vertical migration and feeding in *Sagitta elegans* Verrill. *Ecology* **54:** 300–314.

PIERCE, E. L. 1951. The Chaetognatha of the west coast of Florida. *Biol. Bull.* **100:** 206–228.

RUSSELL, F. S. 1927. The vertical distribution of plankton in the sea. *Biol. Rev.* **2:** 213–262.

RUSSELL, F. S. 1931. The vertical distribution of marine macroplankton. X. Notes on the behavior of *Sagitta* in the Plymouth area. *J. Mar. Biol. Assoc. U. K.* **17:** 391–407.

Reference: *Biol. Bull.* **160:** 431–437. (June, 1981)

BUD SEPARATION IN *HYDRA OLIGACTIS*

BETH L. SERSIG AND GEORGIA E. LESH-LAURIE

Department of Biology, Cleveland State University, Cleveland, Ohio 44115

ABSTRACT

Bud detachment with peduncle and basal disc formation distinguishes the asexual reproductive products of *Hydra* from the secondary axes of colonial coelenterates. This bud individuation was examined in *Hydra oligactis* using three approaches. Isolation experiments showed that parental body column could enhance hypostomal tissue's development of peduncle and basal disc. This established that the parental body column has a role in bud detachment. Grafting procedures used distal budding to indicate ability to direct bud separation. In bud formation and separation from the most distal surface of a subhypostomally amputated parental animal, the budding region of the parental body column was both necessary and sufficient for individuation. Cell quantification studies revealed a combination of three cellular characteristics in regions directing distal budding: a small proportion of nerve cells, a "big" interstitial-cell/epithelial cell ratio considerably <1, and an interstitial-cell derivative/epithelial cell ratio of <0.50.

INTRODUCTION

The freshwater coelenterate *Hydra* reproduces asexually by budding, forming replicate individuals. Among the significant questions raised by budding are the bases for the distinction between a new axis of symmetry that detaches (a hydra bud) and one that does not (leading to the formation of a colonial animal) (Webster, 1971). Hydra budding is usually divided into three distinct stages: initiation, elongation, and separation or individuation. Clarkson and Wolpert (1967) categorized the morphological changes that occur during this process, from a deformation of the parental animal's body column (the bud protrusion) to the elaboration of the mature bud before its detachment.

Grafting experiments between symbiotic and aposymbiotic *Hydra viridis* specimens established that the cell source for buds is at least partly parental. Epithelial cells of the epidermal and gastrodermal layers move down the parent body column and out into the bud (Shostak and Kankel, 1967). Non-epithelial cell types, such as nematocytes and interstitial cells (I-cells), most of which normally migrate distally, move quickly into any developing bud present (Herlands and Bode, 1974). The cellular composition of the developing bud also changes from an early homogeneous distribution of epithelial, interstitial, and nematoblast cells to the pattern of regional specialization characteristic of a nonbudding parent animal (Sanyal, 1967; Bode *et al.*, 1973).

Bud initiation and elongation have been investigated extensively. Bud initation appears to be related to a requisite quantity of parental cells in the budding region (Shostak *et al.*, 1968; Webster and Hamilton, 1972). Inhibiting mitosis and DNA synthesis by radiation does not interrupt the process, suggesting that bud elongation may be a tissue movement phenomenon (Clarkson and Wolpert, 1967). Campbell (1974) described some of the mechanics of this elongation process.

Received 9 November 1980; accepted 6 February 1980.

Bud separation, or individuation, has long been recognized as a distinct event. Rand (1899) recognized a difference between lateral graft separations from a host animal and bud separation. His lateral grafts would eventually grow apart but they lacked the sequence of peduncle and basal disc formation seen in buds. Rand remarked that the conditions affecting bud constriction were a matter of conjecture. Seventy years later Webster (1971) noted the precise control of bud size at detachment, but also was unable to identify the cause of bud separation.

This investigation attempted to identify bud separation properties in *Hydra oligactis* by localizing the source of separation capacity and by describing conditions at that source when it directs bud detachment. Bud separation is accomplished by the formation of peduncle and basal disc; therefore, capacity to develop these structures was analyzed in various situations.

We also investigated the ability of tissues such as the bud hypostomal tissue, parent hypostomal tissue, and/or parental body column to direct distal bud detachment. The influence of these tissues on bud detachment was assessed by challenging them to direct bud separation from the distal region of a host animal, where budding does not normally occur (Webster, 1966; Tardent, 1972; Brooks, 1976).

MATERIALS AND METHODS

Culture methods

H. oligactis were mass cultured in 8" fingerbowls according to the methods of Lenhoff and Brown (1970). The balanced saline solution (M solution) was prepared with distilled water and contained kanamycin sulfate to retard bacterial growth (Lesh-Laurie *et al.*, 1976). Animals were maintained in constant light and temperature (19 ± 1°C) and were fed to repletion each Monday, Wednesday, and Friday with *Artemia salina* nauplii. They were allowed to feed 4–6 h, after which they were cleaned of adherent debris and placed in fresh culture solution.

Experimental procedures

For experimental procedures, *H. oligactis* were taken from mass culture 1 day post-feeding. Several experiments involved excising tissue and then monitoring the isolated piece for peduncle and basal disc formation. Tissue pieces isolated included: a zero-to-two-tentacled bud with parental body annulus, a bud protrusion with parental body column, a zero-tentacled bud with parental body column, a four-tentacled bud with parental body column, parent and bud hypostomes and tentacle rings, and parent and bud axes isolated from a single animal. All tissue isolates were cultured at a density of 0.5 ml culture solution/isolate. Attachment to the container was considered evidence of a basal disc. Where parental body column was isolated with a developing bud, the development of the parental tissue also was noted. Isolate development was followed for up to 16 days.

In grafting, tissue surgically removed from a donor animal was transferred to a host animal. The donor and host tissue then were allowed to heal together. Two petri dishes were used, one of them modified to hold host and donor tissues in apposition during healing. Amputations were performed in one petri dish, which contained culture solution. The prepared host animal was transferred to the second dish, which had been layered with paraffin wax with human hairs embedded vertically in it, and which contained culture solution covering the projecting hairs.

To graft donor tissue to a host distal surface, nonbudding animals, 1 day post-feeding, were selected as hosts and amputated subhypostomally with a single clean cut immediately proximal to the tentacle whorl. The remaining proximal body was transferred to the paraffinized dish. Using watchmaker's #5 forceps, the host was positioned so that a hair was aligned through the axis of the body column.

The desired donor tissue was then excised and transferred to the paraffinized dish. By using forceps that passed through the piece of donor tissue, it was placed in direct apposition to host tissue. Care was taken to place cut surfaces in contact and to align the axes of host and donor. Grafts were held together by an alcohol-cleaned loop of human hair and allowed to heal for 2–4 h. The hair loop then was removed and the graft gently lifted from its position on the hair.

To graft donor tissue to the lateral surface or to the bud of a host animal, the host animal was placed onto the hair so that the hair passed through the parent and/or bud, with the parent lying horizontally on the paraffin surface.

After they were removed from the paraffinized dish, the grafted samples were placed in a 1″ covered culture dish containing 5–7 ml of culture solution. Grafting success ranged from 50%, in grafts involving bud protrusions, to 100%, in grafts employing four-tentacled bud hypostomes or parental hypostomes as donor tissue. Grafted animals were monitored for budding activity for up to 14 days.

Cell counting

Cells were separated and counted using the maceration technique of David (1973). Ten tissue samples of each selected body region were macerated together and spread on a 3 × 1″ glass microscope slide. Cell counts were made by random edge-to-edge sweeps across the short dimension of the slide, thus eliminating any effects of selective cell accumulations at the slide edges.

RESULTS

Peduncle and basal disc formation from isolated bud and parent tissue

Various portions of the parent and bud axes were isolated and monitored to identify the body regions of parents and buds that can form peduncle and basal disc. Table I indicates that: (1) bud tissue forms peduncle and basal disc more readily than does parental tissue (experiments 1–5); and (2) both parental and bud tissue form these structures more readily as the position of amputation moves

TABLE I

Isolation experiments.

Isolate type	Sample size	Number showing peduncle and basal disc formation
1. Parental hypostome and tentacle ring	41	0
2. Four-tentacled bud hypostome and tentacle ring	35	5
3. Two-tentacled bud hypostome and tentacle ring	32	4
4. Parental body column	55	27
5. Bud body column	56	51
6. Zero-to-two-tentacled bud with parental annulus	34	34
7. Bud protrusion with parental annulus	31	31
8. Four-tentacled bud with parental body	31	28
9. Zero-tentacled bud with parental body	35	33
10. Bud protrusion with parental body	44	43

proximally along the body column (experiments 4 and 5). Isolates that included a portion of parental tissue with the bud formed peduncles and basal discs most frequently (experiments 6–10). Thus, parental body column tissue may enhance the ability of a developing bud to form peduncle and basal disc.

Identification of the region which directs bud detachment

A series of grafting experiments examined bud separation more critically. These experiments were designed to identify the tissue directing detachment of a bud, and where along the distoproximal axis of an organism this tissue could exert its influence. We examined principally the ability of tissue to direct "distal budding," or bud formation and detachment from the most distal surface of a parental animal amputated subhypostomally. Distal budding rigorously tests the ability of grafted tissue to direct bud detachment.

Initially, presumptive bud hypostomes and bud hypostomes of various developmental stages were grafted to parental hosts amputated subhypostomally. Definitive bud hypostome tissue did not lead to distal budding: No distal budding was seen in 141 grafts when donor tissue was a four-tentacled bud hypostome, two-tentacled bud hypostome, or zero-tentacled bud hypostome. However, when donor tissue was a bud protrusion with an attached wedge of parental body column, 30 of 56 grafts produced distal budding. In the latter series of grafts, some parental tissue was included due to the small size of the presumptive bud at this early stage. These results suggest a requirement for parental tissue in directing bud detachment. However, bud tissue free from hypostomes also might have been responsible for the effect.

To test whether a large amount of bud tissue, free of bud hypostome, could direct distal budding, a two-tentacled bud axis was used as donor tissue. In none of 33 grafts did a bud axis alone lead to distal budding. A two-tentacled bud axis that also included a parental tissue wedge, however, produced distal budding in 22 of 31 grafts.

To determine if parental tissue alone, rather than parental *and* bud tissue, could direct bud separation from the distal surface of a host amputated subhypostomally, we used portions of parental body column as donor tissue. Parental budding region directed distal budding (9 of 30 cases), while parental gastric region did not (0 of 31 cases).

Influence of parental hypostome on bud development and separation

According to the gradient theory (Weimer, 1928), the dominant parental hypostome and adjacent tissue normally prevent budding from regions more distal than the budding region. Browne (1909) and Li and Yao (1945) found that bud donor tissue grafted to the gastric region of a parental host initiated an axial outgrowth but did not cause the development of a bud that separated. However, Clarkson and Wolpert (1967), grafted an early bud hypostome rudiment to the parental gastric region and produced bud development with separation. Based on these observations and the previous results (where a graft of bud protrusion with attached parental body wedge led to distal budding) we tested whether a bud protrusion with an attached parental wedge could direct bud detachment when grafted laterally in the distal-most gastric region of a parental host (*i.e.* very near the dominant parental hypostome).

In 22 of 32 grafts, the bud completed its development and detached, showing that bud protrusions with parental axial tissue can lead to bud development and separation from a parental host in a region more distal than the budding region, and more closely associated with the parental hypostome. Coincident with bud separation, the parental organism formed a slight lateral protrusion at the base of the developing bud. This remained for the rest of the experiment.

A parental hypostome grafted alone did not lead to formation of a bud that separated from the parental body column, nor did it direct distal budding from a parental animal. To determine if a parental hypostome would prevent separation of a bud which had begun development, a parental hypostome was grafted to a zero-tentacled bud axis that had been amputated proximal to the presumptive hypostomal region. In none of 30 grafts did a parental hypostome interfere with completion of bud development and separation.

Cellular distributions

The grafting experiments indicated that tissue from the budding region of the parental body column was necessary to direct bud detachment. To examine a possible cellular basis for this ability, we macerated tissues from several body regions and identified and categorized the disaggregated cells from each sample. Samples of parental budding region annuli and bud protrusions with parental body column were compared with parental hypostomes (without tentacles), zero-tentacled bud hypostomes, two-tentacled bud hypostomes with tentacles, four-tentacled bud hypostomes with tentacles, one-third annuli of gastric regions, and peduncle annuli.

Table II gives the number of cells of various types in selected body regions, expressed as a proportion of the number of epithelial cells. The percentage of epithelial cells in a bud remains fairly constant throughout its development (Bode *et al.*, 1973). The cell types counted were epithelial cells (epitheliomuscular cells

TABLE II

Cell-type proportions from selected hydra body regions. The epithelial-cell proportion column gives the epitheliomuscular and digestive cell count/total cell count, with raw data in parentheses. In all other columns the proportion is the proportion of the cell type listed to epithelial cells.

Body region	Epithelial cell proportion	Little I-cells	Big I-cells	Nerve cells	Nematoblasts, nematocytes, and nerve cells	Secretory cells
Parental hypostome	0.45 (367/839)	0.16	0.27	0.34	0.56	0.24
Four-tentacled bud hypostome	0.40 (333/841)	0.35	0.41	0.29	0.66	0.11
Two-tentacled bud hypostome	0.50 (400/793)	0.14	0.41	0.14	0.36	0.07
Peduncle	0.83 (604/728)	0	0	0.05	0.12	0.09
⅓ gastric region annulus	0.26 (231/876)	1.13	0.78	0.06	0.76	0.12
⅓ budding region annulus	0.34 (277/807)	0.81	0.65	0.02	0.43	0.03
Bud protrusion with parental body column	0.40 (312/782)	0.42	0.62	0.07	0.44	0.03
Zero-tentacled bud hypostome	0.31 (254/826)	0.59	1.09	0.05	0.49	0.08

and digestive cells), nerve cells, "big" interstitial (I) cells (which encompass the stem cell population and cells which may differentiate into nerves), "little" I-cells (which may differentiate into nematoblasts and ultimately nematocytes), nematoblast cells, nematocytes, and secretory cells (David, 1973).

The proportions of big I/epithelial cells, nerve/epithelial cells, and I-cell derivatives (nematoblasts, nematocytes, and nerves)/epithelial cells were similar in the budding region and bud protrusion. The big I-cell/epithelial proportion always was considerably <1 (0.65 and 0.62 respectively); the nerve cell/epithelial proportions were very low (0.02 and 0.07 respectively), and the I-cell derivatives/epithelial proportions were slightly <0.50 (0.43 and 0.44 respectively). Based on morphological or temporal similarities to the budding region or bud protrusion, gastric region tissue and zero-tentacled bud hypostome also might have been expected to direct distal budding. Each of these regions shared two of the three cellular patterns characterizing the budding region and bud protrusion. The gastric region showed a higher proportion of I-cell derivatives (0.76) than the noted <0.50 level. The zero-tentacled bud showed a high big I-cell proportion (1.09) in its presumptive hypostomal area. Older hypostomal tissue samples contained higher proportions of nerve cells and lower proportions of big I-cells than the samples involved in bud separation.

Discussion

A hydra bud is distinguished from a secondary axis of a colonial coelenterate by its formation of a peduncle and a basal disc, and by its subsequent individuation. From this investigation we conclude that the ability to direct bud separation from a parental axis resides within the parental body column. While various isolated tissues of *H. oligactis*, including isolated bud tissue, can form peduncles and basal discs, bud tissue apparently does not direct the natural separation of a secondary axis from a parental body column.

Hypostomal tissues from bud-protrusion through parental stages were grafted to the distal surface of parental hosts, to test rigorously whether these tissues can direct bud separation. Neither homografts nor heterografts of parental hypostomes resulted in distal budding (Sersig, unpublished). This observation served as a control and in particular indicated that the surgical manipulations alone would not lead to distal budding. Bud hypostomes also did not direct distal budding. However, bud protrusions grafted with small amounts of adjacent parental body column, and subhypostomally amputated two-tentacled bud axis with adjacent parental body column, did lead to bud separation.

These results suggest a requirement for parental body column tissue in directing bud separation. This hypothesis was verified by the observation that parent budding region annuli, but not gastric region annuli, lead to distal budding. Thus, parental body tissue is both required and sufficient for bud separation.

Numerous additional experiments challenged isolated parental and bud tissue to form peduncle and basal disc, structures necessary to complete bud individuation. These data collectively further substantiate an involvement of parental body column in directing bud separation.

A cellular basis for the capacity to direct bud separation also was sought, by comparing regions capable of directing distal budding (budding region and bud protrusion) to regions not implicated in bud separation. A combination of three cellular distributions was unique to the budding region and bud protrusion: considerably fewer big I-cells than epithelial cells, few nerve cells, and an I-cell derivative/epithelial cell proportion slightly <0.50. To assess the significance of these data, each distribution must be evaluated individually and comparisons made between the cell compositions presented here and those in homologous tissue of non-budding hydra mutants (Moore and Campbell, 1973).

ACKNOWLEDGMENTS

The authors thank V. R. Flechtner for her critical reading of this manuscript. This work was supported by a Denver M. Eckert grant for cancer research (VC 248) from the American Cancer Society.

LITERATURE CITED

BODE, H., S. BERKING, C. N. DAVID, A. GIERER, H. SCHALLER, AND E. TRENKNER. 1973. Quantitative analysis of cell types during growth and morphogenesis in hydra. *Wilhelm Roux' Arch. Entwicklungsmech. Org.*, **171:** 269–285.

BROOKS, D. C. 1976. *An analysis of some biosynthetic events of hydrid tentacle morphogenesis.* Ph.D. thesis, Case Western Reserve University, Cleveland, Ohio. 143 p.

BROWNE, E. N. 1909. The production of new hydranths in *Hydra* by the insertion of small grafts. *J. Exp. Zool.*, **7:** 1–24.

CAMPBELL, R. D. 1974. Cell movements in *Hydra. Am. Zool.* **14:** 523–535.

CLARKSON, S. G., AND L. WOLPERT. 1967. Bud morphogenesis in *Hydra. Nature* **214:** 780–783.

DAVID, C. N. 1973. A quantitative method for maceration of hydra tissue. *Wilhelm Roux' Arch. Entwicklungsmech. Org.* **171:** 259–268.

HERLANDS, R. L., AND H. R. BODE. 1974. Oriented migration of interstitial cells and nematocytes in *Hydra attenuata. Wilhelm Roux' Arch. Entwicklungsmech. Org.* **176:** 67–88.

LENHOFF, H. M., AND R. D. BROWN. 1970. Mass culture of hydra: An improved method and its application to other aquatic invertebrates. *Lab. Anim.* **4:** 139–154.

LESH-LAURIE, G. E., D. C. BROOKS, AND E. R. KAPLAN. 1976. Biosynthetic events of hydrid regeneration. I. The role of DNA synthesis during tentacle elaboration. *Wilhelm Roux's Arch. Entwicklungsmech. Org.* **180:** 157–174.

LI, H. P., AND T. YAO. 1945. Studies on the organizer problem in *Pelmatohydra oligactis.* III. Bud induction by developing hypostome. *J. Exp. Biol.* **21:** 155–160.

MOORE, L., AND R. D. CAMPBELL. 1973. Non-budding strains of hydra: Isolation from sexual crosses and developmental regulation of form. *J. Exp. Zool.* **185:** 73–82.

RAND, H. W. 1899. The regulation of graft abnormalities in *Hydra. Arch. Entwick. Mech. Org.* **9:** 161–214.

SANYAL, S. 1967. Cellular dynamics in the morphogenesis of hydra during bud development and regeneration. *Anat. Anz.*, **120:** 1–13.

SHOSTAK, S., J. W. BISBEE, C. ASHKIN, AND R. V. TAMMARIELLO. 1968. Budding in *Hydra viridis. J. Exp. Zool.*, **4:** 423–430.

SHOSTAK, S., AND D. R. KANKEL. 1967. Morphogenetic movements during budding in *Hydra. Develop. Biol.*, **15:** 451–463.

TARDENT, P., 1972. Experimente zum Knospungsprozess von *Hydra attenuata Pall. Rev. Suisse Zool.*, **79:** 355–375.

WEBSTER, G. 1966. Studies on pattern regulation in hydra. II. Factors controlling hypostome formation. *J. Embryol. Exp. Morphol.* **16:** 105–122.

WEBSTER, G. 1971. Morphogenesis and pattern formation. *Biol. Rev.* **46:** 1–46.

WEBSTER, G., AND S. HAMILTON. 1972. Bud initiation in *Hydra:* The role of cell division and cell movement. *J. Embryol. Exp. Morphol.* **27:** 301–316.

WEIMER, B. R. 1928. The physiological gradients in *Hydra.* I. Reconstitution and budding in relation to length of a piece and body level in *Pelmatohydra oligactis. Physiol. Zool.* **1:** 183–230.

INDEX

A

Action of various anticoagulants on hemolymphs of lobsters and spiny lobsters, 55
Adaptive significance of a semilunar rhythm in the terrestrial crab *Sesarma*, 311
Algae: *Avrainvillea* symbiosis with *Costasiella lilianae*, 43
  symbiosis with hydra, 175
Allen, Dennis M., and Wendy B. Allen, Seasonal dynamics of a leech-mysid shrimp interaction in a temperate salt marsh, 1
Allen, Wendy B., see Dennis M. Allen, 1
Ammonia excretion, in prawn *Macrobrachium rosenbergii*, 349
AMP and CMP in *Artemia* diet, 265
Amphibola crenata, snail, oxygen consumption, salinity and hypoxia, 332
Androgenic gland in mole crab *Emerita asiatica*, 161
Annelids: see *Chaetopterus pergamentaceus*, leech
Anticoagulants' effects on hemolymphs of lobsters and spiny lobsters, 55
Aplysia and circadian rhythms, 199
Apomictic parthenogenesis in a hermaphroditic terrestrial slug, *Deroceras laeve* (Müller), 123
Armstrong, D. A., K. Strange, J. Crowe, and M. Simmons, High salinity acclimation by the prawn *Macrobrachium rosenbergii*: uptake of exogenous ammonia and changes in endogenous nitrogen compounds, 349
Arrow worm, see *Sagitta crassa*
Ascoglossa, see *Costasiella lilianae*
Association between *Mytilus chilensis* Hupe (Bivalvia, Mytilidae) and *Edotea magellanica* Cunningham (Isopoda: Valvifera) in Southern Chile, 107
Asterias, see seastars
Atlantic continental shelf, northwest, seastar distribution, 394
Atwood, H. L., see C. K. Govind, 69
Avrainvillea nigricans, see algae

B

Babu, K. Sasira, see T. Pavan Kumar, 114
Back transfer of metabolites from hydra to algae, 175
Barnacle, see *Pollicipes polymerus*
Bellon-Humbert, Chantal, Francois Van Herp, Godfried E. C. M. Strollenberg, and J. Manuel Denucé, Histological and physiological aspects of the medulla externa X organ, a neurosecretory cell group in the eyestalk of *Palaemon serratus* Pennant (Crustacea, Decapoda, Natantia), 11
Bigger, C. H., see I. S. Johnston, 280
Blood: chemistry in deep-sea fishes, 250
  sodium, in prawn *Macrobrachium rosenbergii*, 349
Budd, T. W., see C. K. Govind, 69
Bud separation in *Hydra oligactis*, 431
Butterfly, see *Danaus plexippus*

C

Callyspongia diffusa, sponge, allograft reactions, 280
Carbohydrate rhythms, content in slug *Laevicaulis alte*, 114
Carbon fixation in *Costasiella lilianae*-algae symbiosis, 43
Cellular mechanisms of circadian rhythms, 199
Cercaria adranocerca identified as *Lasiotocus minutus*, 146
Chaetopterus pergamentaceus, ultrastructural analysis of cytoplasmic localization, 228
Chlorella symbiosis with *Hydra*, 175
Chloroplast symbiosis in a non-elysiid mollusc, *Costasiella lilianae* Marcus (Hermaeidae: Ascoglossa (=Sacoglossa)): effects of temperature, light intensity, and starvation on carbon fixation rate, 43
Ciliates, *Favella ehrenbergii* predation on, 136
Cimberg, Robert L., Variability in brooding activity in the stalked barnacle, *Pollicipes polymerus*, 31
Circadian fluctuations in total protein and carbohydrate content in the slug *Laevicaulis alte* (Ferussac, 1821), 114
Circadian timing by endogenous oscillators in the nervous system: toward cellular mechanisms, 199
Clam, see *Gemma gemma*
Clark, Kerry B., Kathe R. Jensen, Hugh M. Stirts, and Cesar Fermin, Chloroplast symbiosis in a non-elysiid mollusc, *Costasiella lilianae* Marcus (Hermaeidae: Ascoglossa (=Sacoglossa)): effects of temperature, light intensity, and starvation on carbon fixation rate, 43
Cloaca in suction in soft-bodied invertebrates, 189
Clocks in circadian timing, 199
CMP and AMP in *Artemia* diet, 265
Cnidarians, see *Pennaria tiarella*
Coelenterata, see *Hydra*
Coelomic pressure in suction in soft-bodied invertebrates, 189
Colchicine, effect on *Pennaria tiarella* development, 303
Collier, J. R., Protein synthesis in the polar lobe and lobeless egg of *Ilyanassa obsoleta*, 366
Commensalism, *Edotea magellanica* with *Mytilus chilensis*, 107

Competition, intraspecific, in sponges and corals, 280
Composition of the blood serum of deep-sea fishes, 250
Coral: see *Montastrea cavernosa, Montipora verrucosa*
Costasiella lilianae, mollusc, 43
Crab, see *Emerita asiatica*
Crassostrea virginica, oyster, reproduction, 80
Crowe, J., see D. A. Armstrong, 349
Crustacea: see *Emerita asiatica, Homarus*, lobsters and spiny lobsters, shrimp
Cytoplasmic localization in *Chaetopterus pergamentaceus*, 228

D

Danaus plexippus, monarch butterfly, reproductive diapause, 89
Daniel, Thomas L. Fish mucus: *In situ* measurements of polymer drag reduction, 376
Deep-sea fishes, composition of blood serum, 250
Denucé, J. Manuel, see Chantal Bellon-Humbert, 11
Deroceras laeve, slug, apomictic parthenogenesis in, 123
DeRosa, Y. Susan, and W. Otto Friesen, Morphology of leech sensilla: observations with the scanning electron microscope, 383
Development: of *Artemia*, 265
  of *Chaetopterus pergamentaceus*, 228
  of *Ilyanassa obsoleta*, 366
  of nervous system in *Pennaria tiarella*, 303
  of *Lasiotocus elongatus*, 155
  of *Lasiotocus minutus*, 146
Diapause, reproductive, in monarch butterfly, 89
Diet purines and pyrimidines and *Artemia* morphogenesis, 265
Digenea: see *Lasiotocus elongatus, Lasiotocus minutus*
Dinoflagellates, predation on by *Favella ehrenbergii*, 136
Distribution patterns of common seastars of the Middle Atlantic continental shelf of the Northwest Atlantic (Gulf of Maine to Cape Hatteras), 394
Drag reduction, by fish mucus, 376
Durliat, Michèle, and Roger Vranckx, Action of various anticoagulants on hemolymphs of lobsters and spiny lobsters, 55

E

Eckberg, William R., An ultrastructural analysis of cytoplasmic localization in *Chaetopterus pergamentaceus*, 228
Edotea magellanica (isopod), commensal with *Mytilus chilensis* (mollusc), 107
Eggs: of *Chaetopterus pergamentaceus*, cytoplasmic localization, 228
  of *Ilyanassa obsoleta*, protein synthesis, 366
Elasmobranchs' blood chemistry and osmoregulation, 250
Electrophoretic patterns of proteins, 55
Embryology of *Ilyanassa obsoleta*, 366
Emerita asiatica, mole crab, protandric hermaphroditism, 161
Energetics of coral *Montastrea cavernosa*, 292
Epigonal organ of Atlantic nurse shark, 240
Escape reaction in arrow worm *Sagitta crassa*, 419
Evolution: of fish osmoregulatory mechanisms, 250
  of slug *Deroceras laeve*, 123
Eyestalk removal in *Palaemon serratus*, 11

F

Factors affecting oxygen consumption in the marine pulmonate *Amphibola crenata* (Gmelin, 1791), 332
Fänge, Ragnar, and Artur Mattisson, The lymphomyeloid (hemopoietic) system of the Atlantic nurse shark, *Ginglymostoma cirratum*, 240
Favella ehrenbergii, tintinnid, prey selection, 136
Feeding, of leech *Haementeria ghilianii*, in laboratory, 322
Fermin, Cesar, see Kerry B. Clark, 43
Fiber composition and innervation patterns of the limb closer muscle in the lobster *Homarus americanus*, 69
Fish mucus: *In situ* measurement of polymer drag reduction, 376
Follicle cells in mole crab *Emerita asiatica*, 161
Food: see predation, starvation, feeding, diet
Franz, D. R., E. K. Worley, and A. S. Merrill, Distribution patterns of common seastars of the Middle Atlantic continental shelf of the Northwest Atlantic (Gulf of Maine to Cape Hatteras), 394
Free amino acids, in prawn *Macrobrachium rosenbergii*, 349
Friesen, W. Otto, see Y. Susan DeRosa, 383

G

Ganglionic cells of *Pennaria tiarella*, 303
Gemma gemma, clam, host for *Lasiotocus*, 146, 155
Genetic analysis and circadian rhythms, 199
Genetics in slug *Deroceras laeve*, 123
Genolopa identified with *Lasiotocus*, 146
Ginglymostoma cirratum lymphomyeloid system, 240
Glossiphoniid leech, see *Haementeria ghilianii*
Gonyaulax, prey of tintinnid *Favella ehrenbergii*, 136
Goto, Taichiro, and M. Yoshida, Oriented light reaction of the arrow worm, *Sagitta crassa* Tokioka, 419
Govind, C. K., T. W. Budd, and H. L. Atwood, Fiber composition and innervation patterns of

the limb closer muscle in the lobster *Homarus americanus*, 69
Griffith, Robert W., Composition of the blood serum of deep-sea fishes, 250
Growth and reproduction of the giant glossiphoniid leech *Haementeria ghilianii*, 322
Guillard, R. R. L., see Diane Stoecker, 136

H

Haementeria ghilianii, leech, growth and reproduction in laboratory, 322
Hayes, Paul F., and R. Winston Menzel, The reproductive cycle of early setting *Crassostrea virginica* (Gmelin) in the northern Gulf of Mexico, and its implications for population recruitment, 80
Hemolymphs of lobsters and spiny lobsters, coagulation, 55
Herman, William S., Studies on the adult reproductive diapause of the monarch butterfly, *Danaus plexippus*, 89
Hermaphroditism: in mole crab *Emerita asiatica*, 161
  in slug *Deroceras laeve*, 123
Hernandorena, A., Metabolic significance of the dietary CMP requirements in relation to *Artemia* morphogenesis, 265
Heterocapsa, prey of *Favella ehrenbergii*, 136
High mobility group peptides in *Ilyanassa obsoleta*, 366
High salinity acclimation by the prawn *Macrobrachium rosenbergii*: uptake of exogenous ammonia and changes in endogenous nitrogen compounds, 349
Hildemann, W. H., see I. S. Johnston, 280
Hill, Burke, Respiratory adaptations of three species of *Upogebia* (Thalassinidea, crustacea) with special reference to low tide periods, 272
Hirundo medicinalis, leech sensilla, 383
Histological and physiological aspects of the medulla externa X organ, a neurosecretory cell group in the eyestalk of *Palaemon serratus* Pennant (Crustacea, Decapoda, Natantia), 11
Histones in *Ilyanassa obsoleta*, 366
Hoffmann, Richard J., see Nancy L. Nicklas, 123
Homarus: *americanus*, fiber composition and innervation of muscle, 69
  vulgaris, hemolymph clotting, 55
Hydra: green, symbiosis with algae, 175
  oligactis, bud separation, 431
  viridis: transfer of metabolites between Hydra and symbiotic algae, 175
Hydrostatic support for suction in invertebrates, 189
Hydrozoans, see *Pennaria tiarella*
Hyperosmotic transfer in prawn *Macrobrachium rosenbergii*, 349
Hypoxia, *Upogebia* adaptations to, 272

I

Ilyanassa obsoleta, development, 366
Immune system of Atlantic nurse shark, *Ginglymostoma cirratum*, 240
Immunological reaction of sponges and corals, 280
Influence of temperature on the kinetics of allograft reactions in a tropical sponge and a reef coral, 280
Inhaling without ribs: the problem of suction in soft-bodied invertebrates, 189
Innervation patterns of *Homarus americanus* muscle, 69
Insect reproduction, monarch butterfly, 89
Interstitial cells in *Pennaria tiarella*, 303
Intertidal ecology, barnacle reproduction, 31
Intraspecific competition in sponges and corals, 280
Ions and circadian rhythms, 199
Isopod, see *Edotea magellanica*

J

Jacklet, Jon W., Circadian timing by endogenous oscillators in the nervous system: toward cellular mechanisms, 199
Jaramillo, Eduardo, Jorge Navarro, and Jürgen Winter, The association between *Mytilus chilensis* Hupe (Bivalvia, Mytilidae) and *Edotea magellanica* Cunningham (Isopoda: Valvifera) in Southern Chile, 107
Jasus lalendei, pallensis, frontalis, spiny lobsters, hemolymph coagulation, 55
Jensen, Kathe R., see Kerry B. Clark, 43
Johnston, I. S., P. L. Jokiel, C. H. Bigger, and W. H. Hildemann, The influence of temperature on the kinetics of allograft reactions in a tropical sponge and a reef coral, 280
Jokiel, P. L., see I. S. Johnston, 280
Juvenile hormone, in monarch butterfly, 89

K

Kavee, Rhonda M., see Diane Stoecker, 136
Knight, A., see D. A. Armstrong, 349
Kramer, Andrew P., see Roy T. Sawyer, 322
Kumar, T. Pavan, R. Ramamurthi, and K. Sasira Babu, Circadian fluctuations in total protein and carbohydrate content in the slug *Laevicaulis alte* (Ferussac, 1821), 114

L

Laevicaulis alte, slug, circadian rhythms, 114
Larval release rhythms in crab *Sesarma*, 311
Lasiotocus: *elongatus*, trematode, 155
  minutus, trematode, 146
Lasker, Howard R., Phenotypic variation in the coral *Montastrea cavernosa* and its effects on colony energetics, 292

Leech: see *Haementeria ghilianii, Hirundo medicinalis, Mysidobdella borealis*
LePont, Francois, see Roy T. Sawyer, 322
Lesh-Laurie, Georgia E., see Beth L. Sarsig, 431
Leucine (protein precursor) in hydra-algae symbiosis, 175
Light: and circadian rhythms, 199
  and *Sagitta crassa*, 419
  effects on *Costasiella lilianae*-algae symbiosis, 43
Life history, developmental stages, and taxonomic relations of the digenetic trematode *Lasiotocus minutus* (Manter, 1931) Thomas, 1959, 146
Life history: of isopod *Edotea magellanica*, 107
  of trematode *Lasiotocus elongatus*, 155
  of trematode *Lasiotocus minutus*, 146
Lobster: hemolymph coagulation, 55
  fiber composition and innervation of muscle, 69
Lunar rhythms in carb *Sesarma*, 311
Lymphomyeloid (hemopoietic) system of the Atlantic nurse shark, *Ginglymostoma cirratum*, 240.

M

Macrobrachium rosenbergii, prawn, salinity acclimation, 349
Margulis, Lynn, see Glyne Thorington, 175
Marsh, salt, leech-shrimp interaction in, 1
Martin, Vicki Joan, and Mary Beth Thomas, The origin of the nervous system in *Pennaria tiarella*, as revealed by treatment with colchicine, 303
Mattisson, Artur, see Ragnar Fänge, 240
Medulla externa X organ of *Palaemon serratus*, 11
Membranes and circadian rhythms, 199
Menidia menidia, host of *Lasiotocus* (trematode), 146, 155
Menzel, R. Winston, see Paul F. Hayes, 80
Merrill, A. S., see D. R. Franz, 394
Metabolic significance of the dietary CMP requirement in relation to *Artemia* morphogenesis, 265
Metabolites, symbiotic exchange from hydra to algae, 175
Microzooplankton, *Favella ehrenbergii* prey selection, 136
Mollusca: see *Costasiella lilianae*, *Mytilus chilensis*, slug, snail.
Monarch butterfly reproductive diapause, 89
Montastrea cavernosa, coral, effects of phenotypic variation, 292
Montipora verrucosa, coral, allograft reactions, 280
Morphogenesis of *Artemia*, and CMP requirement, 265
Morphology: life history, and systematic relations of *Lasiotocus elongatus* (Manter, 1931) Thomas, 1959 (Trematoda: Digenea), 155
  of leech sensilla: observations with the scanning electron microscope, 383
  variation, in coral *Montastrea cavernosa*, 292
Motoneuron of *Homarus americanus*, 69
Muscle of *Homarus americanus*, 69
Mysidobdella borealis, marine leech, interaction with mysid shrimp, 1
Mysid shrimp, see *Neomysis*
Mussel, see *Mytilus chilensis*
Mytilus chilensis, (mussel) occupied by *Edotea magellanica* (isopod), 107

N

Navarro, Jorge, see Eduardo Jaramillo, 107
Neomysis americana, shrimp, association with leech, 1
Neoteny in mole crab *Emerita asiatica*, 161
Nervous system: development in *Pennaria tiarella*, 303
  in *Homarus americanus*, 69
Neurons and circadian rhythms, 199
Neurosecretion of *Palaemon serratus*, 11
Neurosensory cells of *Pennaria tiarella*, 303
Nicklas, Nancy L., and Richard J. Hoffmann, Apomictic parthenogenesis in a hermaphroditic terrestrial slug, *Deroceras laeve* (Müller), 123
Nucleic acid precursors in hydra-algae symbiosis, 175

O

Oriented light reactions of the arrow worm, *Sagitta crassa* Tokioka, 419
Origin of the nervous system in *Pennaria tiarella*, as revealed by treatment with colchicine, 303
Orotic acid (RNA precursor) in hydra-algae symbiosis, 175
Osmoregulation: in fishes, 250
  in prawn *Macrobrachium rosenbergii*, 349
Oscillators in nervous system and circadian timing, 199
Oyster, see *Crassostrea virginica*
Oxygen consumption: and tension, and *Amphibola crenata*, snail in *Upogebia*, 272

P

Palaemon serratus medulla externa X organ, 11
Panulirus, regius, vulgaris, spiny lobsters, hemolymph coagulation, 55
Parasitism, leech-shrimp, 1
Pennaria tiarella, hydrozoan, nervous system, 303
Phenotypic variation in the coral *Montastrea cavernosa* and its effects on colony energetics, 292
Photosynthesis: in coral zooxanthellae, 292
  in *Costasiella lilianae*-algae symbiosis, 43
  in hydra-algae symbiosis, 175
Phototaxis, in *Sagitta crassa*, 419
Pigment control in *Palaemon serratus*, 11

Planula of *Pennaria tiarella*, 303
Pollicipes polymerus, barnacle, brooding, 31
Population recruitment in *Crassostrea virginica*, 80
Prawn, see shrimp
Predation, selective, by Tintinnid *Favella ehrenbergii* on dinoflagellates, 136
Proctotrema identified with *Lasiotocus*, 146
Protandric hermaphroditism in a mole crab, *Emerita asiatica* (Decapoda: Anomura), 161
Protein: electrophoretic and immunologic patterns, in lobster hemolymph, 55
  rhythms in content in slug *Laevicaulis alte*, 114
  synthesis and circadian rhythms, 199
  synthesis in *Artemia*, 265
  synthesis in the polar lobe and lobeless egg of *Ilyanassa obsoleta*, 366
Protozoa, *Favella ehrenbergii* predation, 136
Pulmonate: *Amphibola crenata*, effect of salinity and oxygen tension on respiration, 322
  Deroceras laeve, apomictic parthenogenesis in, 123
Purines, dietary, 265
Pyrimidines, dietary, 265

R

Races, physiological, of barnacles, 31
Ramamurthi, R., see T. Pavan Kumar, 114
Reproduction: asexual, in *Hydra*, 431
  cycle in oyster *Crassostrea virginica*, 80
  ecology of in barnacles, 31
  of leech *Haementeria ghilianii*, 322
  of mole crab *Emerita asiatica*, 161
  of monarch butterfly, 89
Reproductive cycle of early setting *Crassostrea virginica* (Gmelin) in the northern Gulf of Mexico, and its implications for population recruitment, 80
Respiration: in *Amphibola crenata*, effects of salinity and low oxygen tension, 322
  in coral, 292
Respiratory adaptations of three species of *Upogebia* (Thalassinidea, Crustacea) with special reference to low tide periods, 272
Rhythms: circadian, 199
  circadian, in slug *Laevicaulis alte*, 114
  semilunar, in crab *Sesarma*, 311
RNA synthesis in *Artemia*, 265

S

Sacoglossa, see *Costasiella lilianae*
Sagitta crassa, phototaxis, 419
Saigusa, Masayuki. Adaptive significance of a semilunar rhythm in the terrestrial crab *Sesarma*, 311
Salinity: effect on *Amphibola crenata* respiration, 332
  effect on *macrobrachium rosenbergii*, 349
Salmo gairdneri, drag reduction by mucus, 376
Sarsig, Beth L., and Georgia E. Lesh-Laurie, Bud separation in *Hydra oligactis*, 431
Sawyer, Roy T., Francois LePont, Duncan K. Stuart, and Adrew P. Kramer, Growth and reproduction of the giant glossiphoniid leech *Haementeria ghilianii*, 322
Scanning electron microscopy of leech sensilla, 383
Seasonal dynamics of a leech-mysid shrimp interaction in a temperate salt marsh, 1
Seastars, distribution in Northwest Atlantic, 394
Selective predation by *Favella ehrenbergii* (Tintinnia) on and among dinoflagellates), 136
Semilunar rhythm in crab *Sesarma*, 311
Sensory receptors of leech, 383
Serum in fishes, 250
Shark, see *Ginglymostoma cirratum*
Shrimp: see *Macrobrachium, Neomysis, Palaemon, Upogebia*
Shumway, Sandra E., Factors affecting oxygen consumption in the marine pulmonate *Amphibola crenata* (Gmelin, 1791), 332
Simmons, M., see D. A. Armstrong, 349
Slug: see *Costasiella lilianae, Deroceras laeve, Laevicaulis alte*
Snail: see *Amphibola crenata, Ilyanassa obsoleta*
Spiny lobsters, hemolymph coagulation, 55
Spleen of Atlantic nurse shark, *Ginglymostoma cirratum*, 240
Sponge, *Callyspongia diffusa*, allograft reactions, 280
Starvation, effects on *Costasiella lilianea*-algae symbiosis, 43
Stirts, Hugh M., see Kerry B. Clark, 43
Stoecker, Diane, R. R. L. Guillard, and Rhonda M. Kavee, Selective predation by *Favella ehrenbergii* (Tintinnia) on and among dinoflagellates, 136
Strange, K., see D. A. Armstrong, 349
Strollenberg, Godfried E. C. M., see Chantal Bellon-Humbert, 11
Stuart, Duncan K., see Roy T. Sawyer, 322
Studies on the adult reproductive diapause of the monarch butterfly, *Danaus plexippus*, 89
Stunkard, Horace W.: The life history, developmental stages, and taxonomic relations of the digenetic trematode *Lasiotocus minutus* (Manter, 1931) Thomas, 1959, 146
  The morphology, life history, and systematic relations of *Lasiotocus elongatus* (Manter, 1931) Thomas, 1959 (Trematoda: Digenea), 155
Subramoniam, T., Protandric hermaphroditism in a mole crab, *Emerita asiatica* (Decapoda: Anomura), 161
Suction in soft-bodied invertebrates, 189
Symbiosis: in coral *Montastrea cavernosa*, 292
  Costasiella lilianae-algae, 43
  Hydra viridis-algae, 175

T

Target-aiming behavior in arrow worm *Sagitta crassa*, 419

Taxonomy: of trematode *Lasiotocus elongatus*, 155
  of trematode *Lasiotocus minutus*, 146
Teleosts, blood chemistry and osmoregulation, 250
Temperature: effects on barnacle reproduction, 31
  effects on *Costasiella lilianae*-algae symbiosis, 43
  effects on sponge and coral allograft reactions, 280
THOMAS, MARY BETH, see Vicki Joan Martin, 303
THORINGTON, GLYNE, AND LYNN MARGULIS, *Hydra viridis*: transfer of metabolites between *Hydra* and symbiotic algae, 175
Tide: adaptations of *Upogebia* at low, 272
  and rhythms of crab *Sesarma*, 311
Tintinnids: *Favella ehrenbergii* prey selection, 136
Tracer studies in hydra-algae symbiosis, 175
Trematoda, see *Lasiotocus*
Trout, see *Salmo gairdneri*
Two-dimensional electrophoresis, 366

U

Ultrastructural analysis of cytoplasmic localization in *Chaetopterus pergamentaceus*, 228
Upogebia, shrimp, respiration, 272
Urechis caupo, innkeeper worm, suction, inhalation, 189

V

VAN HERP, FRANCOIS, see Chantal Bellon-Humbert, 11
Variability in brooding activity in the stalked barnacle *Pollicipes polymerus*, 31
VRANCKX, ROGER, see Michèle Durliat, 55

W

WINTER, JÜRGEN, see Eduardo Jaramillo, 107
WOLCOTT, THOMAS G. Inhaling without ribs: the problem of suction in soft-bodied invertebrates, 189
WORLEY, E. K., see D. R. Franz, 394
Worm, innkeeper, see *Urechis caupo*

X, Y, Z

YOSHIDA, M., see Taichiro Goto, 419
Zooxanthellae in coral *Montastrea cavernosa*, 292

Continued from Cover Two

high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

4. ***A condensed title*** or running head of no more than 35 letters and spaces should appear at the top of the title page.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text sould be headed LITERATURE CITED, and must be typed double spaced on separate pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin.* Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; the most recent issue). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Visindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger*, *Ecology*, *Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature*, *Science*, *Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** *The Biological Bulletin* has no page charges. However, authors will be requested to help pay printing charges of manuscripts that are unusually costly due to length or numbers of tables, figures, or formulae. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates or foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than correction of printers' or editors' errors).

CONTENTS

ARMSTRONG, D. A., K. STRANGE, J. CROWE, A. KNIGHT, AND M. SIMMONS
High salinity acclimation by the prawn *Macrobrachium rosenbergii*: uptake of exogenous ammonia and changes in endogenous nitrogen compounds 349

COLLIER, J. R.
Protein synthesis in the polar lobe and lobeless egg of *Ilyanassa obsoleta* 366

DANIEL, THOMAS L.
Fish mucus: *In situ* measurements of polymer drag reduction 376

DEROSA, Y. SUSAN, AND W. OTTO FRIESEN
Morphology of leech sensilla: observations with the scanning electron microscope 383

FRANZ, D. R., E. K. WORLEY, AND A. S. MERRILL
Distribution patterns of common seastars of the Middle Atlantic continental shelf of the Northwest Atlantic (Gulf of Maine to Cape Hatteras) 394

GOTO, TAICHIRO, AND M. YOSHIDA
Oriented light reactions of the arrow worm *Sagitta crassa* Tokioka 419

SARSIG, BETH L., AND GEORGIA E. LESH-LAURIE
Bud separation in *Hydra oligactis* 431

INDEX TO VOLUME 160 438

DEC [illegible]

MBL/WHOI LIBRARY
WH 1B1X 9